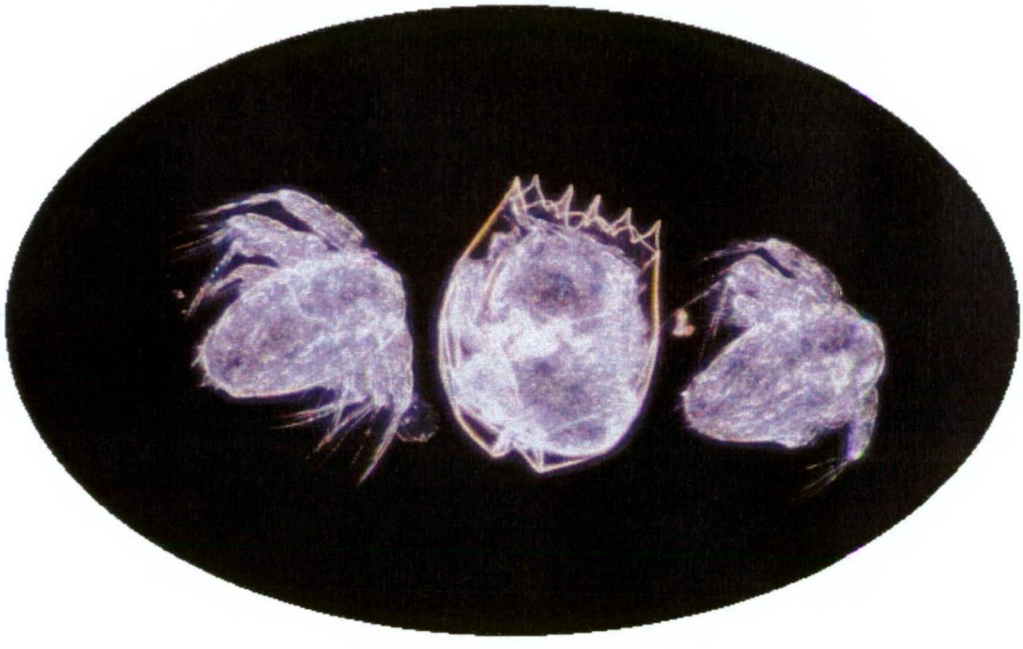
The culture of copepods as live food for marine fish larvae



Acartia species nauplii (Copepoda) flanking *Brachionus rotundiformus* (Rotifera)

by

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Declaration

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Abstract

The diversification of marine finfish aquaculture has identified a requirement for live foods other than the traditional rotifer and brine shrimp species. As copepods constitute a natural component of the diets of larval fish, three species were isolated and their life cycle, environmental tolerances, and productivities investigated. Preliminary trials were also conducted feeding them to larval finfish.

The temperate harpacticoid *Tisbe* species and the tropical cyclopoid *Apocyclops dengizicus* were chosen for these trials as a result of their persistence in aquaculture environs and apparent tolerance of a wide range of environmental conditions. Both *Tisbe* sp. and *A. dengizicus* populations in culture were found to tolerate temperatures and salinities over the ranges of 10 °C to 35 °C and 25 ‰ to 70 ‰ respectively, and were well accepted by first feeding larvae of test fish species. *Tisbe* sp. populations maintained at 20 °C and 35‰ and fed a mixed algal diet of *Tetraselmis* sp. and *Isochrysis galbana* at a density of $1.6x10^5$ cells mL^{-1} exhibited a mean generation time of 6.5 days. The maximum culture density recorded was 6,000 *Tisbe* L^{-1}. *A. dengizicus* culture populations exhibited a mean generation time of 5.3 days when cultured at 29-32 °C and 25 ‰, with a maximum culture density of 4,500 individuals L^{-1} achieved when fed the same mixed algal diet as *Tisbe* sp..

Trials conducted with *Tisbe* sp. and larval greenback flounder (*Rhombosolea tapirina*) showed larvae ingested *Tisbe* sp. nauplii in preference to rotifers (*Brachionus plicatilis*) at initiation of feeding. Developing flounder larvae targeted progressively larger copepod stages consuming ovigerous *Tisbe* sp. at 12 days post-hatch. Barramundi (*Lates calcarifer*) larvae were shown to be successful predators of all *Apocyclops dengizicus* life stages with ovigerous *A dengizicus* ingested by larvae at 6 days post-hatch. Only moribund barramundi larvae were preyed on by the cyclopoid.

The tropical calanoid *Acartia* species was selected for these trials on the basis of the reported success achieved in Thailand when larval red snapper (*Lutjanus argentimaculatus*) were reared on zooplankton cultures containing *Acartia* spp..

Acartia sp. cultures, although tolerant of temperatures and salinities between 23 °C and 35 °C and 5 ‰ and 45 ‰ respectively, exhibited an increase in numbers only when maintained at 35‰. An estimated mean generation time of seven days was observed at 30°C. The maximum culture density achieved was 1,200 individuals L^{-1} when *Acartia* sp. were fed a mixed algal diet comprising *Isochrysis galbana, Rhodomonas* sp., *Tetraselmis* sp. and *Heterocapsa neie* in the ratio 4:2:1:0.1 providing a final algal density of $2.3x10^6$ cells mL^{-1}.

Feeding trials conducted with *Acartia* sp. and golden snapper (*Lutjanus johnii*) in volumes less than 100 L proved unsuccessful, however semi-extensive trials conducted in 40 m^3 tanks yielded larval survival rates of 40 % compared with less than 0.1 % achieved when rotifers were used (Schipp, Bosmans & Marshall, 1999).

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"If we knew what we were doing,
then it wouldn't be called research would it?"
Albert Einstein

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General Introduction

1.1 The need for an alternative live food

Prompted by declining wild fish stocks and increased demand for fresh fish the diversification of aquaculture has been fuelled by global population growth and recognition of the health benefits associated with regular consumption of fresh fish (Zamora, 1990). However the expansion of aquaculture into the culture of marine finfish species has been accompanied by difficulties associated with obtaining acceptable levels of larval survival, growth, and morphological normality (Witt et al., 1984; Sorgeloos et al., 1995; Schipp et al., 1999; Marte, 2001).

Attempts to rear larvae of marine finfish have met with significant difficulties globally regarding rates of growth and survival comparable to those attainable with freshwater-estuarine species under culture conditions (Witt et al., 1984; Sorgeloos et al., 1995). The major bottleneck for aquaculture continues to be the reliable supply of quality larvae and juveniles to stock grow-out production systems (Støttrup et al., 1986; Sorgeloos et al., 1995; Battaglene & Fielder, 1997; Toledo et al., 1999). Larval diets have been identified as the major limiting factor; most marine fish with aquaculture potential possessing limited yolk reserves, small mouths and primitive digestive systems at the commencement of exogenous feeding (Bromage & Roberts, 1995; Kolkovski, 2001).

The ultimate aim of some sectors of the aquaculture industry is the replacement of live foods with off-the-shelf products in the production of commercial species (Fernández-Diaz et al., 1994; Sorgeloos et al., 1995). The development of an artificial feed capable of sustaining larval growth and survival rates comparable to those achieved with live foods would reduce labour and space requirements making aquaculture more resource efficient. However to date this has been limited in its success.

The Australian Fisheries Research and Development Corporation (FRDC), whilst recognising the value of aquaculture and the deficiencies in the current hatchery feeds available, supports the development of a co-ordinated approach between industry and research organisations in the study of foods for larval finfish. To this end, a workshop on Hatchery Feeds was convened in Cairns, in March 2000. The workshop recognised the progress being made in the development of artificial microdiets for the replacement of live foods, however the then available diets have failed to yield comparable survival and growth responses in high value aquaculture species. While work continues refining microdiet technologies to address the issues of ingestion and digestion in association with nutritional requirements and culture system design (Southgate & Kolkovski, 2000) live food organisms continue to form the basis of finfish larval production for the stocking of commercial grow-out facilities.

Since one of the more limiting factors in larval nutritional physiology seems to be the absence of a differentiated stomach, it has been suggested more research should be devoted to the question why live food can be utilised efficiently without a stomach but formulated feeds cannot (Segner et al., 1993). Copepods have been associated with improved development of the larval digestive tract post-metamorphosis in halibut (Luizi et al., 1999), which may explain improved resilience of copepod-fed fish to the rigors of metamorphosis and weaning.

The use of wild zooplankton, comprising mainly copepods, has been consistently associated with improved survival, growth, stress tolerance and expression of typical morphological features of many marine fish larvae including: turbot, mahi mahi, yellow-fin seabream, grouper, golden snapper, red seabream, red snapper, halibut, black seabream and wolf fish (Kuhlmann et al., 1981; Lee et al., 1981; Kraul, 1983; Witt et al., 1984; Ringo et al., 1987; Naas, 1990; Kraul et al., 1992; Chang & Lei, 1993; Norsker & Støttrup, 1994; Sunyoto et al., 1995; Abu-Rezq et al., 1997; Doi et al., 1997a; Doi et al., 1997b; Rønnestad et al., 1998; Schipp et al., 1999; Støttrup, 2000; Bell et al., 2001).

Numerous developments have taken place since the initial work was undertaken into artificial microdiets for zooplankton with the intention of replacing algae and later as a delivery mechanism for elements for enrichment (Jones et al., 1974; Walford & Lam, 1987; Southgate & Kolkovski, 2000). During the 1970's, research into alternatives to live foods for larval fishes resulted in the development of microdiets of an appropriate size which continued to improve in terms of stability in the water column and the degree of nutrient leaching. Problems still remain with respect to larval acceptance, digestibility, stability in the water column and nutritional completeness for some species (Nellen, 1985; Dabrowski & Culver, 1991; Munilla-Moran et al., 1990; Clawson & Lovell, 1992; Fernández-Diaz et al., 1994; Zambonino Infante & Cahu, 1994) although advances in microdiet formulations such as liposomes have the potential to alleviate some of the shortcomings by facilitating the incorporation of water soluble elements such as free amino acids, water soluble vitamins and pigments into the aqueous volume, or lipid soluble nutrients such as essential fatty acids into the phospholipid encapsulating membrane (Koven et al., 1999; Rønnestad et al., 1999; Southgate, 2000; Kolkovski, 2001).

It is recognised that suitable artificial diets are unlikely to be available in the near future, so there is presently a need to develop a system for the reliable production of an alternative live food source for marine fish larvae (Fujita, 1973; Støttrup et al., 1986; Person-Le Ruyet et al., 1993; Sorgeloos et al., 1995).

Table 1.1.1: Nomenclature of finfish species referred to in the text of this thesis. Scientific names follow Eschmeyer (1998). * indicates the common name used in the body of this thesis.

Family	Valid species name	Commonest synonym in literature	Common name
Abarhichadidae	*Anarhichas lupus* (Linnaeus, 1758)		wolf fish
Centropomidae	*Lates calcarifer* (Bloch, 1790)		{*barramundi { seabass
Chanidae	*Chanos chanos* (Forsskäl, 1775)		milkfish
Clupidae	*Clupea harengus* (Linnaeus, 1758)		herring
Clupeidae	*Clupea sagax* (Jenyns, 1842)	*Sardinops sagax*	Pacific sardine
California	Perca saxatilis (Walbaum, 1792)	*Morone saxatilis*	striped bass
Coryphaenidae	*Coryphaena hippurus* (Linnaeus, 1758)		{*mahi mahi { dolphin fish
Glaucosomaidae	*Glaucosoma hebraicum* (Richardson, 1845)		dhufish
Latridae	*Latris lineata* (Schneider, 1801)		striped trumpeter
Lutjanidae	*Scieana argentimaculata* (Forrskäl, 1775)	*Lutjanus argentimaculatus*	{*red snapper { mangrove jack
Lutjanidae	*Anthias johnii* (Bloch 1972)	*Lutjanus johnii*	golden snapper
Moronidae	*Perca labrax* (Linnaeus, 1758)		seabass
Mugilidae	*Mugil cephalis* (Linnaeus, 1758)		grey mullet
Pleuronectidae	*Pleuronectes americanus* (Walbaum, 1792)		winter flounder
Pleuronectidae	*Pleuronectes hippoglossus* (Linneaus, 1758)	*Hippoglossus hippoglossus*	halibut
Pleuronectidae	*Pleuronectes platessa* (Linneaus, 1758)		plaice
Pleuronectidae	*Pleuronectes yokohamae* (Günther, 1877)	*Limanda yokohamae*	{*mud dab { marbled sole

Table 1.1.1: (Continued) Nomenclature of finfish species referred to in the text of this thesis. Scientific names follow Eschmeyer (1998). * indicates the common name used in the body of this thesis.

Family	Valid species name	Commonest synonym in literature	Common Name
Pleuronectidae	*Rhombosolea tapirina* (Günther, 1862)		greenback flounder
Scophthalmidae	*Pleuronectes maximus* (Linneaus, 1758)	*Scophthalmus maximus*	turbot
Salmonidae	*Salmo salar* (Linneaus, 1758)		Atlantic salmon
Sciaenidae	*Perca ocellata* (Linneaus, 1758)	*Scianops ocellata*	red drum
Serranidae	*Bola coioides* (Hamilton 1822)	*Epinephelus coioides*	red-spotted grouper
Serranidae	*Cromileptes altivelis* (Valenciennes, 1828)		barramundi cod
Serranidae	*Epinephelus lanceolatus* (Bloch, 1790)		giant grouper
Serranidae	*Perca fuscoguttatus* (Forrskäl, 1775)	*Epinephelus fuscoguttatus*	{*brown-marbled grouper { flowery cod
Serranidae	*Perca tauvina* (Forrskäl, 1775)	*Epinephelus tauvina*	grey grouper
Serranidae	*Plectropomus maculatus* (Bloch, 1790)		coral trout
Siganidae	*Chaetodon guttatus* (Bloch, 1787)	*Siganus guttatus*	rabbitfish
Sillaginidae	*Sillaginodes punctatus* (Gill, 1862)		King George whiting
Soleidae	*Pleuronectes solea* (Linneaus, 1758)	*Solea solea*	sole
Soleidae	*Solea senegalensis* (Kaup, 1858)		Senegal sole
Sparidae	*Acanthopagrus cuvieri* (Day, 1875)		sobaity
Sparidae	*Chrysophrys major* (Temminck & Schegel, 1843)	*Pagrus major*	red seabream
Sparidae	*Chrysophrys schlegelii* (Bleeker, 1854)	*Acanthropagrus schlegelii*	black sea bream
Sparidae	*Pagrus latus* (Richardson & Solander, 1842)	*Pagrus auratus*	pink snapper
Sparidae	*Sparus aurata* (Linnaeus, 1758)		gilthead seabream

Traditionally, aquaculture techniques involve the use of rotifers (*Brachionus* spp.) and brine shrimp (*Artemia*) as live foods (Hoff & Snell, 1993; Evjemo & Olsen, 1997; Hagiwara et al., 2001), however diversification of the finfish species for use in aquaculture has been associated with problems ranging from poor survival and growth of larvae (Kuhlmann et al., 1981; Naas, 1990), to morphological and pigmentation abnormalities (Fukusho et al., 1980; Næss et al., 1995; Cobcroft et al., 2001). The use of copepods in larval diets has resulted in improved larval survival, growth, health, pigmentation and resistance to the stresses of culture conditions (Kuhlmann et al., 1981; James & Al-Khars, 1984; Witt et al., 1984; Ringo et al., 1987; Kraul et al., 1992; Mourente et al., 1993; Harboe et al., 1994; Sorgeloos et al., 1995; Sunyoto et al., 1995; Doi et al., 1997b; Rønnestad et al., 1998; Schipp et al., 1999).

The potential value of copepods to the culture of marine finfish provided the motivation for my research. The Australian aquaculture industry is expanding at a rapid rate and expressed a requirement for live food alternatives to rotifers and *Artemia* as these were not successful in the larviculture (i.e. rearing of larvae) of some 'new' marine species. My research was conducted under the umbrella of the CRC for Aquaculture funded jointly by Australian industry and government. The following introduction provides the background to the value of copepods as a live food in terms of larval fish requirements, the information available relating to the culture of copepod species and the means by which copepod culture success can be measured. Each copepod species-specific chapter commences with a genera-specific introduction. The final chapter discusses my findings in the context of the potential of the three copepod species as live food organisms in the Australian aquaculture industry.

The use of copepods as a live food in Australian aquaculture is relatively recent, the culture of copepods essentially unknown to the majority of aquaculturists. The development of culture 'recipes' to minimise the inconvenience to aquaculturists and maximise the value of copepods as a live food is the primary motivation behind the research conducted.

1.2 Criteria for larval finfish diets

The early life of a fish is characterised by extensive morphological and functional differentiation involving respiration, swimming muscles and swimming mode, sense organs, energy metabolism, enzyme systems and digestive physiology (Hunter, 1981; Osse, 1990; Segner et al., 1993; De Silva & Anderson, 1995). As a consequence of limited physical development of finfish larvae at the commencement of exogenous feeding (Hunter, 1981; Bromage & Roberts, 1995), marine finfish larvae rely on food selection, mechanical digestion and intestinal enzymes to compensate for the lack of gastric enzymes (Watanabe & Kiron, 1994). Therefore the diets offered to larval finfish must be of exceptionally high nutritive

value and meet a number of criteria to achieve commercially viable levels of survival and growth.

According to Dabrowski (1984), the primary criterion for the selection of a larval food should be biochemical composition, however it is recognised that the problem is more complex. Initiation of larval feeding is not achieved solely through chemical stimuli, but also a combination of visual characteristics (Checkley, 1982; Blaxter, 1986; Tucker, 1992; Utne-Palm, 1999).

A number of studies have suggested an ideal larval feed should be of:

- appropriate size (Ikeda, 1973; Kraul, 1983; Tucker, 1992; De Silva & Anderson, 1995; Liao et al., 2001),
- appropriate nutritional composition (Kraul, 1983; Tucker, 1992; Watanabe & Kiron, 1994; Rønnestad et al., 1999; Liao et al., 2001),
- visible and attractive (Tucker, 1992; Utne-Palm, 1999),
- palatable and digestible (Tucker, 1992; De Silva & Anderson, 1995; Kolkovski, 2001),
- occupy the same habitat as the larvae (Kraul, 1983),
- be stable until eaten (Tucker, 1992), and
- pure (De Silver & Anderson, 1995), that is free of biological and chemical contaminants.

The presentation of diets, either artificial or natural, that fail to meet the above criteria may result in poor larval survival rates, if not 100% mortality. A scarcity of suitable planktonic prey organisms at the onset of exogenous feeding can result in body tissue autolysis and eventual death (Bagarinao, 1986; Segner et al., 1993; Watanabe & Kiron, 1994).

In addition to the above attributes, larval diets should also be easily procurable, reproducible and economical (Nellen, 1985; Tucker 1992; Watanabe & Kiron, 1994; De Silva & Anderson, 1995).

Another attribute of a live food, not often specifically stated, is that the live food organism should not demonstrate any aggressive characteristics such as aggravation of fish larvae, or act as a vector for parasites or pathogens.

Traditional larviculture methods have relied on brine shrimp, *Artemia* spp., alone or in combination with the rotifer *Brachionus plicatilis* (Støttrup et al., 1986; Nanton & Castell, 1998; Kolkovski, 2000). However, nutritional inadequacy combined with the inappropriate size of rotifers and *Artemia* have been identified

as major factors affecting larval survival (Watanabe et al., 1983; Witt et al., 1984; Ohno & Okamura, 1988).

The composition of *Artemia*, and to some extent rotifers, has been shown to be extremely variable depending on the place of origin and its diet, being frequently low in the essential fatty acids (Watanabe et al., 1983; Witt et al., 1984; Ohno & Okamura, 1988). Either or both the long chain fatty acids eicosapentaenoic acid (EPA, 20:5 *n*-3) and docosahexaenoic acid (DHA, 20:6 *n*-3) are essential nutrients for those marine fish species for which essential fatty acid requirements have been determined (Castell, 1979; Robin, 1995). More recently arachadonic acid (AA, 20:4n-3) has also been recognised as an essential fatty acid (Sargent et al., 1999; Izquierdo et al., 2000). A nutritional problem arises because rotifers and *Artemia* usually are deficient in at least one of these fatty acids, and possibly other nutrients (Watanabe et al., 1983; Sargent et al., 1999).

Enrichment techniques focusing on the modification of rotifer and *Artemia* lipid composition have significantly improved survival, growth and pigmentation of larvae from a number of species (Sorgeloos et al., 1988; Kraul, 1993). However, further expansion of aquaculture into tropical species has been associated with additional difficulties not necessarily directly related to fatty acid composition but most probably other attributes such as particle size, digestibility, nutritional composition and other unspecified characteristics exhibited by the natural live foods of the fish species in question (Lokman, 1993; Singhagraiwan & Doi, 1993; Doi et al., 1997; Shansudin et al., 1997; Schipp et al., 1999).

Despite improvements with respect to the development of fatty acid enrichments, such as emulsions, for rotifers and *Artemia*, superior larval performance has been a characteristic of larvae reared on copepod diets. Better results in terms of growth and survival (Witt et al., 1984; Nellen, 1985; Naas, 1990) and the development of pigmentation in the larvae of mud dab (Fukusho et al., 1980), turbot, (Witt et al., 1984; Urup, 1994) and Atlantic halibut (Næss et al., 1995) have also been observed in studies where wild collected plankton have been used. Kraul et al. (1991) found larval mahi mahi survive better under common culture stresses such as high stocking density, disease outbreak, cold weather or metamorphosis when reared on a diet of copepods.

Enriched *Artemia* and rotifers, and wild collections of copepods from halibut hatcheries identified a higher total lipid content for rotifers and *Artemia*, however a higher proportion of essential fatty acids were present in copepods (Evjemo & Olsen, 1997).

In addition to the inappropriate size range and often doubtful nutritional composition of *Artemia*, the supply of *Artemia* is also uncertain, and with reduced supply and increasing demand, the cost of *Artemia* is also increasing (Nellen, 1985; Ohno & Okamura, 1988; Kolkovski, 2000; De Wolf & Candreva, 2001).

Aquaculture therefore is faced with a need to find an interim solution to the lack of both a suitable artificial feed and a live food alternative to rotifers and *Artemia*.

Table 1.1.2: An explanation of the acronyms and symbols used in the text of the thesis.

Acronym	Explanation
‰	parts per thousand, ppt
Ø	diameter
µ	micro, $1x10^{-6}$
AA	the fatty acid arachadonic acid, whose standardised biochemical label is 20:4*n*-3
CRC	Co-operative Research Centre
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DAC	Darwin Aquaculture Centre, Fisheries Division, Northern Territory Department of Business Industry and Resource Development
DHA	the fatty acid docosahexaenoic acid, whose standardised biochemical label is 22:6*n*-3
dph	age of fish larvae given as the number of days post-hatch
EPA	the fatty acid eicosapentanoic acid, whose standardised biochemical label is 20:5*n*-3
EFA	essential fatty acids – defined as those fatty acids which cannot be synthesised in sufficient quantities to meet metabolic demands and therefore must be obtained from the diet
FAA	free amino acids
HUFA	highly unsaturated fatty acids – defined as fatty acids having a carbon number equal to or greater than 20 and a number of double bonds equal to or greater than 3
PVC	poly vinyl chloride - plastic

1.3 Why choose copepods?

As early as 1920, Lebour reported copepod nauplii as a dominant prey in the gut contents of larval fishes caught at sea (May, 1970) and of laboratory-reared larvae fed mixed prey (Detwyler & Houde, 1970), a theme consistent through time (Hunter 1981; Checkley, 1982; Kahan et al., 1981; Kraul, 1983; Norsker & Støttrup, 1994; Shaheen et al., 2001).

Several factors contribute to the success of copepod species as live foods for marine finfish larvae: size range, fatty acid content, amino acids, vitamins, associated enzymes, movement patterns and the subsequent feeding stimulus (Nellen, 1985; Jenkins, 1987; Munilla-Moran et al., 1990; Dabrowski & Culver, 1991; Clawson & Lovell, 1992; Tucker 1992; Fyhn et al., 1993; Zambonino

Infante & Cahu, 1994; Utne-Palm, 2000). This section will discuss these key issues in more detail.

1.3.1 Size

Prey size dominates prey selection patterns of larval fishes (Hunter, 1981; Tucker, 1992), with the optimal prey size for marine fish larvae related to mouth size (Shirota, 1970; Hunter, 1981). The preferred prey size of larvae quickly increases as mouth size and feeding competence increases with optimal prey-size being species-specific (Shirota, 1970; Ghan & Sprules, 1993; Fernández-Diaz et al., 1994). The width of prey is considered the most important factor in selection (Detwyler & Houde, 1970; Govoni et al., 1986). Prey width, not prey length, limits the size of the food organisms ingested (Ghan & Sprules, 1986).

With widths in the range of 50 to 100 µm the mouths of marine larvae (Nellen, 1985; Watanabe & Kiron 1994; Iglesias et al., 1994) tend to be much smaller than their estuarine-freshwater counterparts traditionally reared on rotifers and *Artemia* (Støttrup et al., 1986).

The twelve life stages of a copepod represent a number of discrete particle sizes covering a wide range. For example, the temperate harpacticoid *Tisbe* has nauplii which range from 40-120 µm, while the copepodid stages range from 150-1200 µm (Witt et al., 1984), extending either side of that available with rotifers (50 to 200 µm) and *Artemia* (420 to 8000 µm) (Bromage & Roberts, 1995).

Size alone is not the only issue as red snapper larvae possess a mouth width of 200 µm however small strain rotifers of 100 µm in width were not ingested until 2 to 4 days after the commencement of feeding (Doi & Singhagraiwan, 1993).

Turbot larvae aged 3 days post-hatch were found to have empty stomachs when fed rotifers and *Artemia*, however up to five nauplii were found in the stomachs of turbot larvae when fed a copepod diet (Witt et al., 1984). The smaller size of the copepod nauplii (some less than 50µm) appears to be the underlying reason, not the higher DHA content of the copepods.

Polo et al. (1992) presented gilthead seabream larvae with two size strains of *Brachionus plicatilis* (BS strain: 92-176 µm and S-1 strain: 140-276 µm) and found that larvae performed significantly better when presented with both strains than when presented with either strain in isolation. Both strains exhibited the same proximate chemical composition.

Various size fractions of copepods facilitate the presentation of similar nutritive value packages in various sizes so as to optimise energy return for energy expenditure by larvae. The nutritive value of larger prey can be illustrated by considering the relationship between width of copepods and their weight. Hunter (1981) calculated for an increase of 2.5x in copepod width produced an order of

magnitude increase in dry weight therefore a larva feeding on copepodids 200 µm in width would have to capture ten times the number of prey to obtain the same ration as those feeding on copepodids 500 µm in width.

1.3.2 Nutritional composition

A comparison of the nutritional values of rotifer, brine shrimp and copepod as diets for marine finish larvae highlighted the superiority of copepods as live food organisms (Watanabe et al., 1983; Witt et al., 1984; Støttrup et al., 1986; Kraul et al., 1991; Sargent et al., 1999; Toledo et al., 1999; Evjemo et al., 2001). Copepods are also more dense than the other species in organic material for equivalent wet weight (Szyper, 1989; Han et al., 2001; Sorgeloos et al., 2001) representing a greater nutritional return on energy expended by feeding larvae. The following pages elaborate on the compositional attributes of copepods which render them a nutritionally complete live food suitable for larviculture.

1.3.2.1 Fatty acid content

Finfish larvae reared on diets deficient in the correct highly unsaturated fatty acids (HUFA) exhibit reduced growth, high mortality rates, an increase in the occurrence of physical abnormalities and susceptibility to stress (Castell, 1979; Dhert et al., 1991; Watanabe, 1991; Kanazawa, 1991; Ako et al., 1991; Bisbal, 1991). The deleterious effects of such fatty acid deficiencies on growth and survival are understandable when the physiological role of these compounds is considered.

Fatty acids are critical to the integrity of cells, being major constituents of cell membranes and regulators of permeability (Castell, 1979; Bell et al., 1986; Ederington et al., 1995; Sargent et al., 1999). HUFA also act as important energy sources, are fundamental to the functioning of certain membrane bound enzymes and prostaglandin production, and are essential to normal brain and nerve activity (Castell, 1979; Kanazawa, 1991; Mourente et al., 1993; Watanabe & Kiron, 1994).

Abnormalities in the pigmentation of mud dab larvae have been attributed to a 22:6*n*-3 (docosahexaenoic acid, DHA) deficiency resulting in an inability to transfer information from the retina to the nerve cortex to release the melanocyte stimulating hormone which induces melanin formation responsible for pigmentation (Kanazawa, 1991). The communication failure between the retina and nerve cortex is attributable to incomplete neural development as a direct consequence of DHA deficiency. An insufficiency of DHA in marine fish larval diets is likely to impair neural and visual development with significant, if not, serious consequences for a wide range of physiological and behavioural processes including those dependent on neuroendocrines (Navarro et al., 1997; Sargent et al., 1999).

Sorgeloos et al. (1988) tested the *n*-3 highly unsaturated fatty acid enrichment products Selco® with seabass, barramundi, seabream, mahi mahi, milkfish and

rabbitfish and concluded that both survival and growth were improved; survival was found to be related to EPA (20:5*n*-3), and growth to DHA (22:6*n*-3).

The mortality in larvae can be reduced by replacing nutrient deficient diets as demonstrated in grey grouper by Dhert et al. (1991). The performance of gilthead seabream and grey grouper has also been related to the total *n*-3 HUFA levels, with high DHA:EPA levels proven essential to larval growth and development in both species (Dhert et al., 1991; Mourente et al., 1993).

Susceptibility to stress by mahi mahi can be reduced by using DHA-boosted *Artemia* as a food source with the accompanying advantage of being able to stock the fish at higher density with a greatly reduced risk of disease outbreak. In addition, the fish exhibit greater resistance to cold weather and the rigours of metamorphosis (Ako et al., 1991; Kraul et al., 1991; Kraul et al., 1993).

The yolk sac supply of DHA is rapidly exhausted during early larval stages and is associated with rapid development of the neural system (Mourente et al., 1993). Diets high in *n*-3 HUFA with a high DHA content contribute to an improved development of the nervous system which includes greater visual acuity, enhancing the ability of larvae to feed (Sargent et al., 1999). The net energy gained by the larvae is subsequently expressed in growth rate, survival and stress resistance (Ako et al., 1991; Mourente et al., 1993; Sorgeloos et al., 1995). Koven et al. (2001) also found AA to be beneficial to improved survival as a result of increased stress tolerance by gilthead seabream.

Reasons for the predominance of *n*-3 HUFA in marine finfish, particularly temperate species, may be related to an improved ability to maintain cell membrane fluidity and function with increasing salinity, and pressure, and reduced temperatures (Castell, 1979; Bell et al., 1986, Lovell, 1989). The *n*-3 HUFA level allows a greater degree of unsaturation than *n*-6 fatty acids (Lovell, 1989) necessary to maintain flexibility and permeability characteristics at low temperatures. Studies on warm water species suggest that the importance of lipids lies in their use as an energy source during rapid embryonic development (Southgate et al., 1994). Lipid content of larval barramundi decreased by 51.5% between days 1 and 8 post-hatch while protein decreased by 4.3% - lipid being the major source of energy as is the case with other warm water fishes including mahi mahi, red drum and Senegal sole (Southgate et al., 1994).

Both *n*-3 and *n*-6 fatty acid series are important in freshwater fishes with linolenate (18:3 *n*-3) and linoleate (18:2 *n*-6) satisfying the essential fatty acids requirements for the two series of fatty acids. Marine finfish express an absolute requirement for all three long chain HUFA (AA, EPA and DHA) as they lack the necessary elongation and desaturation enzymes to modify other dietary fatty acids (Castell, 1979; Bell et al., 1986; Lovell, 1989; Watanabe, 1991; Craig et al., 1995; Robin, 1995; Nanton & Castell, 1999).

Tucker (1992) suggested that copepods could also benefit from enrichment on the basis of a 3.4% difference in survival of mud dab fed either unadulterated or HUFA-enriched baker's yeast copepods. However survival rates of 93.8% ±3.32% (± standard deviation) and 97.2± 0.28 % respectively with no differences in the percentage of mal-pigmentation ~1% in both instances (Fukusho et al., 1980) suggests that enrichment may not be worth the effort. Watanabe et al. (1978) reported the 20:5*n*-3 and 22:6*n*-3 essential fatty acid composition of *Tigriopus* was high irrespective of diet culture media. Wild caught *Acartia* also demonstrated high levels of essential fatty acids. Pagano & Saint-Jean (1993) found the biochemical composition of *Acartia clausi* in a tropical lagoon remained stable despite significant variation in environmental conditions.

The ratio of DHA:EPA in larval diets is a determining factor in the health of juvenile fish (Clawson & Lovell, 1992; Dhert, 1991; Kanazawa, 1991; Kraul et al., 1991; Mourente et al. 1993; Watanabe, 1991; Han et al., 2001). The importance of DHA to larval development is indicated by its abundance in the lipid composition of the yolk sac and the fact that unfed larvae exhibit an extremely strong retention of DHA in comparison to other fatty acids.

Tucker (1992) indicates that a dietary *n*-3 HUFA content of 2-4%, including at least 1.0% DHA and 1.0% EPA, should satisfy or exceed the requirements of most marine fish larvae. Rotifers and *Artemia* generally require enrichment to meet these criteria. More recently it has been noted that a ratio of DHA:EPA >2 resulted in the elimination of malpigmentation from halibut (Reiten et al., 1994 IN Bell et al., 2001). *Eurytemora* and *Tisbe furcata* exhibited natural ratios of 2 and 2.2 respectively compared to the maximum of 1.3 achieved with *Artemia* enriched with fish oils high in DHA and EPA (Bell et al., 2001).

As *n*-3 HUFA are essential to normal development of marine finfish larvae the relative content of *n*-3 HUFA may prove an invaluable indicator of the suitability of live food organisms for use in the commercial rearing of marine finfish species.

A significant difference in HUFA content occurs between rotifers, *Artemia* and copepod species (Witt et al., 1984; Nellen, 1985; Watanabe, 1991; Evjemo et al., 2001). The fatty acid composition of copepod nauplii was found to resemble that of newly hatched turbot larvae (Witt et al., 1984; Person-Le Ruyet et al., 1993) both being high in DHA:EPA and *n*-3 HUFA. Differences in lipid profiles of the three feed organisms are also reflected in the survival rates associated with larvae fed rotifers, brine shrimp or copepods. Survival rates increase from rotifer-based diets to recently hatched-*Artemia* diets with greatest survival achieved with larvae fed on copepod nauplii (Witt et al., 1984).

Evjemo & Olsen (1997) investigating the total lipid and *n*-3 HUFA content of the copepods *Temora longicornis* and *Eurytemora*, rotifers and enriched *Artemia* found

that the copepods contained twice the amount of the essential fatty acids than enriched *Artemia*. Watanabe et al. (1978) found that *Tigriopus* sp. copepodids had a high proportion of essential fatty acids regardless of their diet whereas rotifers and *Artemia* were not as nutritious unless their diet was nutritionally replete with the appropriate fatty acids.

Kraul et al. (1993) observed greater stress resistance in mahi mahi when fed copepods than enriched *Artemia* despite the slightly higher DHA level in the enriched *Artemia*. As mahi mahi fed copepods had higher tissue levels of DHA than those fed enriched *Artemia* they postulated that the form of the HUFA in copepods in combination with the slightly better amino acid nutrition offered by copepods may have led to higher incorporation of DHA. Subsequent research has shown *Artemia* to retroconvert DHA to EPA when starved (Navarro et al., 1999; Han et al., 2001) which may also account for the lower levels of DHA in *Artemia*-fed mahi mahi.

1.3.2.2 Amino acid content

Growth is primarily an increase in body muscle mass by protein synthesis and accretion (Rønnestad et al., 1999); any dietary imbalance in the influx of dietary amino acids to the body free amino acid pool may cause increased protein degradation and decreased growth efficiency (Verreth & Segner, 1995). Fish larvae exhibit a high growth rate which correlates to a high dietary requirement for amino acids to cater for the energy needs of the developing embryo for biosynthesis, homeostasis and activity (Watanabe & Kiron, 1994; Verreth & Segner, 1995; Southgate et al., 1994; Rønnestad et al., 1999). Fyhn (1987) suggested that the free amino acids of an organism would serve as an indicator of its suitability as a live food for larvae.

Free amino acids (FAA) are an important component of larval fish diets, initially on the basis of their importance as an energy source during egg and yolk sac stages and in their role in intracellular osmoregulation (Fyhn, 1989; Fyhn et al., 1993; Watanabe & Kiron 1994). The use of FAA to maintain embryonic energy production beyond the yolk-sac stage implies that FAA need to be supplied prior to the larval gut becoming fully functional (Rønnestad et al., 1999). In addition to their physiological role, FAA may act as feeding stimulants for first feeding larvae (Koven et al., 1999). Prey may be ingested on the basis of chemo-attraction from amino acids and other metabolites emanating from the prey organism (Watanabe & Kiron, 1994; Tucker 1992). The success achieved with copepod diets may therefore be attributed in part to the large pool of intracellular FAA they contain (Fyhn & Serigstad, 1987; Dabrowski & Culver, 1991; Fyhn et al., 1995) and the elevated levels of dissolved amino acids in seawater where copepods are abundant (Poulet et al., 1991).

The FAA of calanoid copepods is more than twice the amount of FAA per gram of wet mass than *Artemia* (Næss et al., 1995). Even the freshwater cyclopoid *Cyclops strenuous* was found to have higher free amino acid levels than fasting *Artemia* (Dabrowski & Rusiecki, 1983). Live food organisms regulate cell volume via manipulation of the intracellular pool of FAA. An increase in culture salinity corresponding to increased osmotic pressure is countered by an increase in live food FAA content (Rønnestad et al., 1999).

The protein content of copepods is found to be equal to, or greater than, *Artemia* and rotifers, although this can be influenced by rotifer and *Artemia* source and history. The amino acid balance in copepods also tends to be consistently good regardless of culture conditions, however starving zooplankters will be depleted of protein (Szyper, 1989; Tucker, 1992). Conceicão et al. (1997) found the high levels of free methionine present in natural zooplankton dominated by *Acartia grani* to be largely responsible for the greater growth rates of turbot fed natural zooplankton than that observed for turbot fed *Artemia*.

1.3.2.3 Vitamin content

Vitamin deficiencies in the diet of larval fishes generally manifest themselves as deformities. The importance of the vitamins A, B, C and E are generally accepted (Watanabe & Kiron, 1994; Dedi et al., 1998; Rønnestad et al., 1998; Moren et al., 2001) with various lipid enrichment emulsions incorporating Vitamin E to reduce oxidation of the fatty acids and Vitamin C to increase levels of ascorbic acid (Sorgeloos et al., 2001).

Although HUFA are essential for growth and development, they impose a peroxidation burden on the fish which can result in damage to cell membranes, inactivation of enzymes and damage to genetic material and other vital cell components (Mourente et al., 1999). To maintain health and prevent oxidation-induced lesions and mortalities there must be an effective antioxidation system operating in the fish involving dietary micronutrients such as vitamins E and C or carotenoids (Mourente et al., 1999).

Marine fish are predominantly visual feeders (Hunter, 1981) and therefore they require a functional retina at the onset of first feeding. Animals cannot synthesise vitamin A (retinal) *de novo* and a source must be available to them if retinal function is to be achieved (Rønnestad et al., 1998). The high carotenoid content (from which vitamin A is derived) of copepods is well recognised (Kraul, 1983; Nellen, 1985; Kibria et al., 1997).

Vitamin A has also been implicated in the occurrence of malpigmentation (Kanazawa, 1991; Venizelos & Benetti, 1999). For example, halibut fed a diet of natural zooplankton exhibited no sign of mal-pigmentation in comparison to those fed *Artemia* (Rønnestad et al., 1998).

Excesses of vitamin A are, however, toxic to fish larvae (Rønnestad et al., 1998; Moren et al., 2001). An unidentified form of vitamin A found in *Artemia* was unable to be utilised by fish larvae which indicated a potential for *Artemia* to be associated with toxic effects of the vitamin. The halibut fed enriched *Artemia* exhibited 50 to 80% lower weight specific content than those fed the calanoid *Temora longicornis* (Rønnestad et al., 1998).

In general, Rønnestad et al. (1998) found feeding *Artemia* to larvae resulted in a lower larval vitamin A content compared with halibut fed on copepods. The vitamin A components from the copepod *Temora longicornis* did not include the unidentified form and were incorporated in the larvae more efficiently with no evidence of potential toxic effects (Rønnestad et al., 1998).

Structural malformations appear rapidly as supplies of Vitamin C (ascorbic acid) are rapidly exhausted due to high growth rates of larvae. Vitamin C is an important micronutrient in fish necessary for the synthesis of collagen essential to the formation of connective tissues and bone matrix (Gapasin et al., 1998). The decrease in the body concentrations of ascorbate was relatively slow in larvae fed on live food (Watanabe & Kiron, 1994).

Copepods therefore represent a balanced and non-toxic source of essential vitamins available for use by rapidly growing finfish larvae.

1.3.3 Attractiveness, palatability and digestibility

There still remain some undefined elements which render copepods superior to the traditional rotifer and *Artemia* live foods, which in turn are superior to the readily available microdiets (Person-Le Ruyet, 1989; Dabrowski & Culver, 1991). These elements may be chemical growth factors, or olfactory or visual stimuli.

Food size and movement could be the primary feeding stimulants for early larvae (Hunter, 1981; Meyer, 1986). For some fish in which smell develops first, olfactory stimulants may be more effective at an earlier stage than gustatory stimulants (Tucker, 1992).

In natural ecosystems, prey preference/selectivity may be influenced by visibility, capturability, fine-scale distribution of predator and prey, and innate preference of the predator (Checkley, 1982). Characteristics significant in prey visibility include shape, pigmentation, contrast, movement and size (Govoni et al., 1986; Meyer, 1986; Jenkins, 1987; Blaxter, 1988; Van der Meeren, 1991; Doi et al., 1997; Utne-Palm, 2000).

Copepods successfully stimulate feeding responses in larval fishes (Utne-Palm, 1999). Larval fish collected from the Baltic Sea were found to target copepods from mixed wild zooplankton (Rajasilta & Vuorinen, 1983). Where prey was abundant, larval fishes selected strongly for a preferred species of calanoid

copepod. Tropical lutjanids and grouper also demonstrated a strong selectivity for copepod nauplii in preference to more abundant rotifers, actively targeting copepod nauplii present at densities less than 0.1 mL^{-1} in preference to rotifers present at densities in excess of 5 mL^{-1} (Doi et al., 1997a; Doi et al., 1997b; Ali et al., 1998; Toledo et al., 1999).

Prior to the development of gastric glands, fishes rely on mechanical digestion, food selection and intestinal enzymes to compensate for the lack of gastric enzymes (Watanabe & Kiron, 1994). The role of zooplankton proteases as activators of fish zymogens is well recognised, as is the direct contribution of proteolytic activity to the autolytic processes of food organisms (Dabrowski, 1984; Pedersen, 1984; Nellen, 1985; Person-Le Ruyet, 1989; Tucker, 1992; Munilla-Moran et al., 1990; Kibria et al., 1997).

Lumenal digestion in fish larvae, which relies on the exogenous and/or endogenous digestive enzymes, seems to be the major route of nutrient accretion (Dabrowski & Culver, 1991; Segner et al., 1993). Low specific activities of digestive enzymes have been considered responsible for the poor growth and survival of sole larvae fed formulated diets compared to the performance of juvenile sole fed live diets (Zambonino Infante & Cahu, 1994).

The capacity of larval finfish to absorb lipid increases with development in live food-fed larvae while it is delayed in larvae fed formulated diet (Izquierdo et al., 2000). Segner et al. (1993) suggests the poor survival observed for larvae fed artificial diets in comparison to live food is attributable to the limiting factors of under-developed nutritional physiology of larvae which are compensated for by attributes of live foods such as associated enzymes.

Munilla-Moran et al. (1990) found proteolytic enzyme levels in adults of the copepod *Euytemora hirundoides* exhibited a broad pH optimum range of 5.6 to 9.2, compared with the more restricted and lower levels of those associated with *Artemia* (maximum activity at pH 7.0 and 9.2). Pedersen (1984) examined digestion in first-feeding herring larvae hatched in the laboratory and found that copepods from wild zooplankton samples passed more quickly through the gut and were more thoroughly digested than *Artemia*. Similarly, Luizi et al. (1999) reported more advanced digestive tract development for copepod-fed Atlantic halibut larvae compared to those reared on enriched *Artemia*.

The challenge of analytically and biologically unravelling those components which are responsible for the excellent dietary value of copepods for marine larval fish, remains as the ultimate goal (Sorgeloos et al., 1995, Kibria et al., 1997).

1.3.4 Stability and purity

As outlined in 1.4.2, the chemical composition of copepods in terms of their nutrient density, essential fatty acids, amino acid availability, and proximate

composition has been shown to be superior to that of the more easily cultured rotifer and *Artemia,* even when the latter two are enriched (Kraul et al., 1991; Pagano & Saint-Jean, 1993; Quinn, 1993; Norsker & Støttrup, 1994; Evjemo & Olsen, 1997). Copepods are denser than rotifers and *Artemia* in organic material and also retain their nutritive value for longer periods when starved (Szyper, 1989) which is likely to occur during larviculture.

The benefits to larval survival, growth, normal development and stress resistance achieved by the inclusion of wild harvest copepods in larval diets are offset by the disadvantages of wild zooplankton which are expensive and time consuming to collect, seasonally affected by the level of nutrients in the water (in terms of the species and quantities available), and have the potential to introduce pollutants, predators, parasites, toxins and disease agents into fish cultures (May, 1970; Kahan et al., 1982; Nellen, 1985; Støttrup et al., 1986; Person-Le Ruyet, 1989; Tucker, 1992; Quinn, 1993, Kibria et al., 1997; Evjemo & Olsen, 1997). The mixed nature of the wild harvested copepods also renders controlled experimentation difficult (May, 1970).

Extensive zooplankton culture and harvest have also been associated with production difficulties based on seasonality and intrusion by plankton predators. The use of nutrient enriched treated sewage waters is also associated with disease and chemical contamination of larval rearing systems. Despite these drawbacks the nutritional balance, in particular fatty acid composition, remains highly desirable (Quinn, 1993).

The fertilisation of ponds to enhance phytoplankton blooms is practiced, and the development of methods favourable to minimum input zooplankton culture have received considerable attention (Opuszynsk et al., 1984; Shirgur, 1989; Ohno et al., 1990). The use of quality fertilisers free of heavy metals and coarse filtration of natural waters during the filling of ponds, have been adopted to limit the possibility of adverse impact of pollutants and possible introduction of predators.

The development of continuous culture systems for marine copepods remains the only way to obtain a reliable supply of mono-specific, contaminant-free copepods of consistent quality for research and culture programs for marine finfish (Kahan, 1981; Støttrup et al., 1986; Sun & Fleeger, 1995).

1.4 An introduction to the Copepoda

The aquatic crustaceans comprising the Copepoda inhabit waters ranging from fresh through to hypersaline, temperature extremes from Arctic and Antarctic to the tropics, from heights of 5,540 m in the Himalayan mountains, to 10,002 m depths in the Philippine Trench (Huys & Boxshall, 1991). Copepods are not only free-living but also associated with plants and animals, both as symbionts and as parasites (Huys & Boxshall, 1991).

Members of the subclass Copepoda (*sensu* Bowman & Abele, 1982; Huys & Boxshall, 1991) are readily distinguished from other crustaceans (which all possess two pairs of antennae) despite their morphological plasticity, as all copepods have at least one stage with the following characteristics:

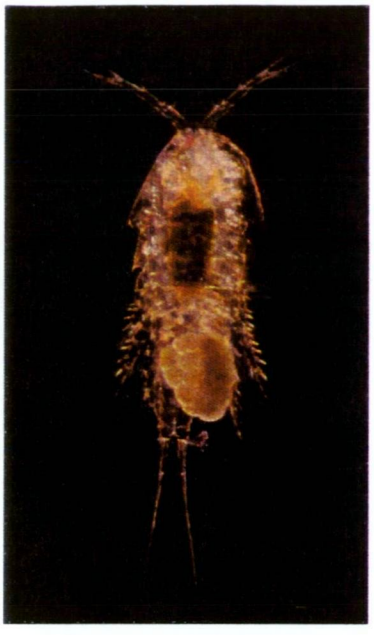
- lack of abdominal appendages,
- possession of at least two pairs of swimming legs (periopods), [an intercoxal sclerite which is an autapomorphy (unique derived feature) of copepods ensures simultaneous beating during locomotion],
- a cephalosome into which the maxilliped-bearing, first thoracic somite is incorporated, and
- the presence of uniramous antennules of up to 27 articles (an autapomorphy of copepods).

Species from three of the ten recognised orders within Copepoda: Harpacticoida, Cyclopoida and Calanoida, are free-living and comprise a natural component of the diet of many finfish species (Hunter 1981; Kraul, 1983). These three orders may be distinguished on the basis of three criteria summarised in Table 1.4.1 (after McLaughlin, 1980; Hicks & Coull, 1983; Wells, 1988) and they are illustrated here by three representative species from north Australia (Figure 1.4.1).

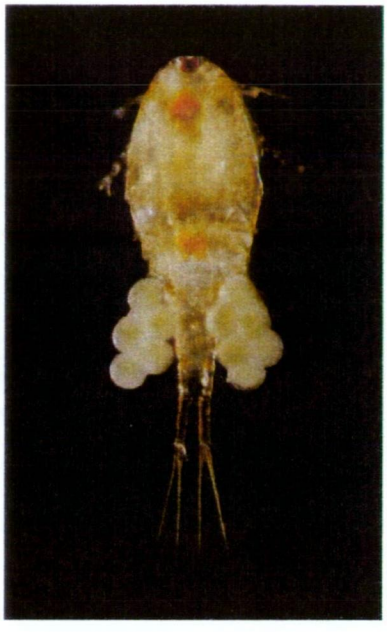
Table 1.4.1: Characteristics distinguishing members of the three free-living orders of the Copepoda (McLaughlin, 1980; Hicks & Coull, 1983; Wells, 1988).

Order	Number of antennule articles	Position of articulation between post-cephalosome segments	Antennule structure
Harpacticoida	<10	5 and 6	usually biramous
Cyclopoida	10-22	5 and 6	uniramous
Calanoida	Usually >22	6 and 7	biramous

The Harpacticoida comprises forty-seven families, the members of which are primarily benthic, and they inhabit both fresh and marine waters. The Cyclopoida which encompasses twelve families include the most abundant and successful copepods in freshwater systems. The members of the Calanoida are the dominant copepods in marine systems and they encompass forty families (Huys & Boxshall, 1991).



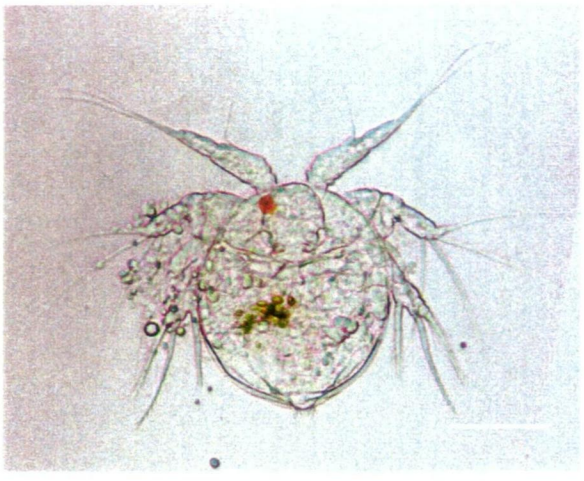
Harpacticoida



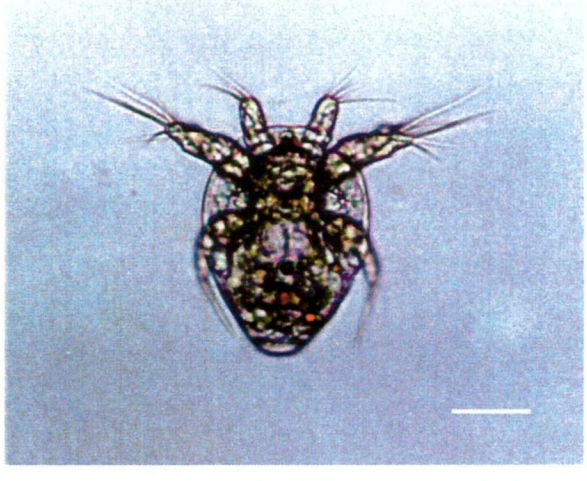
Cyclopoida

Calanoida

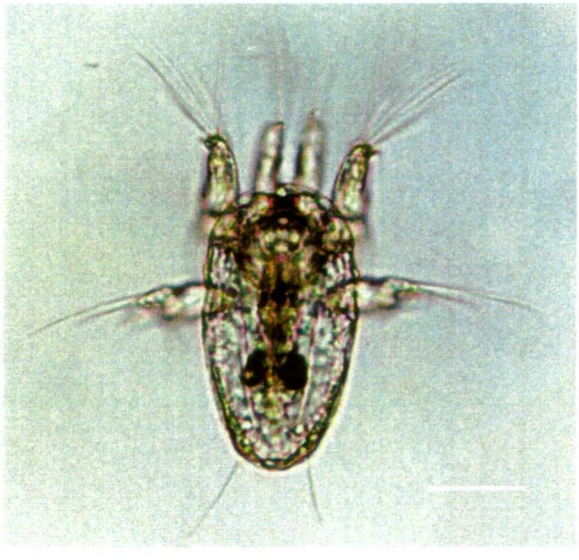
Figure 1.4.1: North Australian representatives from three free-living orders of Copepoda: Harpacticoida –*Euterpina acutifrons*, Cyclopoida-*Apocyclops dengizicus*, and Calanoida - *Acartia* sp.
Scale bars indicate 100 µm.



Tisbe species - Harpacticoida



Apocyclops dengizicus - Cyclopoida



Acartia species - Calanoida

Figure 1.4.2: Early stage nauplii (NI) of the three copepod species investigated. Scale bars are 50 µm.

Copepods exhibit obligate heterosexuality which is associated with the development of sexually dimorphic features to assist in the transfer of a spermatophore from a mature male to a receptive female. Parthenogensis is known to occur in some freshwater harpacticoids, but is not known to occur in any marine copepods (Hicks & Coull, 1983; Wells, 1988).

The life cycle of a copepod typically includes six nauplius stages (NI through NVI) and six copepodid stages (CI through CVI), the sixth copepodid stage corresponds to the sexually mature adult (Wells, 1988; Hart, 1990). As with all other arthropods, copepods grow by size increments at each ecdysis (Longhurst, 1986) and they exhibit metamorphic development with the young hatching from external eggs as first stage nauplii possessing only three pairs of appendages (antennules, antennae and mandibles), but lacking external body segmentation (Figure 1.4.2). Additional segments are added during successive naupliar stages (McLaughlin, 1980; Longhurst, 1986; Wells, 1988). Post-naupliar development consistently follows a metamorphic moult from the last nauplius stage into the first copepodid stage with the resultant form resembling more closely that of the adult (Hart, 1990). Additional somites and appendages are added during successive copepodid stages, culminating in the sexually mature adult. More detail on copepod life cycle and development will be provided for each of the genera studied in subsequent chapters.

Another feature of copepod life cycles is the expression of dormancy. Dormancy is a state of suppressed development manifesting either as diapause or quiescence - diapause being defined as arrested development which is triggered by environmental factors, but is compulsory and ultimately genetically determined; quiescence being a facultative state of retarded development which is induced by adverse environmental conditions (Grice & Marcus, 1981; Dahms, 1995). The phenomenon of dormancy is known only from the free-living Harpacticoida (one freshwater genus and two marine genera), Cyclopoida (all known genera to date are freshwater) and Calanoida (eleven freshwater genera and eleven marine genera, plus three brackish water genera including *Acartia*) (Dahms, 1995).

In copepods, dormancy can occur in various developmental stages. Dormancy has been observed as resting eggs, arrested larval development, juvenile and adult encystment, or arrested development of non-encysted copepodids or adults. Only members of the Calanoida (including species of *Acartia*) are known to produce resting eggs in marine and brackish waters (Dahms, 1995).

There is a latitudinal tendency for dormancy; it prevails in the higher latitudes and decreases towards the tropics. The majority of resting eggs produced by calanoids are released in autumn (Belmonte & Puce, 1994; Dahms, 1995). The production of resting eggs is also more prevalent in coastal rather than oceanic waters (Grice & Marcus, 1981).

Resting eggs are often morphologically dissimilar to subitaneous eggs (i.e. those that hatch without delay) as is the case with *Acartia josephinae*. Scanning electron microscopy of subitaneous eggs revealed the apparently smooth chorion to be tubercular with many small protuberances (Belmonte & Puce, 1994). In contrast, resting eggs of *A. josephinae* has long thick spines whose apical parts were multi-branched (Belmonte & Puce, 1994). A similar morphology was observed for the Mediterranean calanoid *Pontella mediterranea* (Santella & Ianora, 1990).

Environmental cues for the production of resting eggs include photoperiod, temperature, anoxia, desiccation, female age, food availability and high population density (Grice & Marcus, 1981; Ban & Minoda, 1994; Dahms, 1995).

Advantages afforded to copepods by dormancy are:

- co-existence with species occupying the same environmental niche (Dahms, 1995),
- persistence through periods of environmental conditions unfavourable for development, growth and reproduction (Grice & Marcus, 1981; Dahms, 1995),
- predator avoidance (Dahms, 1995), and
- synchronised emergence to promote reproductive success (Grice & Marcus, 1981; Dahms, 1995).

The mechanisms underlying dormancy are as yet undefined (Dahms, 1995). However Ban & Minoda (1994) were able to induce diapause egg production in laboratory cultures of *Eurytemora affinis* at densities of 500 individuals L^{-1}. The transfer of copepods from sparsely populated cultures into water from the dense culture populations resulted in the production of diapause eggs at low copepod densities.

1.5 Culture of copepods

Motivated by curiosity, early naturalists pioneered the culture of copepod species (Barr, 1969). As early as 1910, Allen & Nelson maintained *Calanus finmarchicus* for a number of days in the laboratory. However, it was not until sixty years later that the first successful multiple generation culture of *Calanus* was described (Paffenhöfer & Harris, 1979). In 1923, Murphy cultured the cyclopoid *Oithona nana*, while the benthic harpacticoid (*Euterpina acutifrons*) has been cultured since 1936 (Paffenhöfer & Harris, 1979). Of the three major free-living copepod orders, members of the Calanoida have long been regarded as the most difficult to maintain in culture (Parrish & Wilson, 1978).

The desire to quantify the energy and chemical flows within ecosystems (Zhang & Uhlig, 1993; Pagano & Saint-Jean, 1993; Hutchins et al., 1995), and to understand

recruitment to wild fisheries stock (Kiørboe et al., 1985; Saiz et al., 1996) has lead to an increased interest in copepod culture. Some species have been considered suitable for ecophysiological, toxicology and genetics studies because of their short life cycles, high fecundity and tolerance of environmental fluctuations (Battaglia, 1978; Fava & Crotti, 1979; Milou, 1992; Smith et al., 1994; Williams & Jones, 1994; Dahms & Schminke, 1995; Sibly et al. 2000).

More recently, research on the laboratory rearing of copepods has focused on the development of culture techniques for the production of copepods for use as live foods in aquaculture (Uhlig, 1984; Støttrup et al., 1986; Ohno & Okamura, 1988; Marshall, 1993; Lokman, 1994; Ohno et al., 1996, Schipp et al., 1999).

The reliable and predictable production of sufficient quantities of copepods with minimal resource input remains a challenge (Sun & Fleeger, 1995; Støttrup et al., 1986; Sorgeloos et al., 1995). Some success has been achieved in the Northern Hemisphere with the use of specialised intensive systems using *Acartia tonsa* in the culture of herring, turbot and plaice (Støttrup et al., 1986; Urup, 1994), and in Asia with the use of extensive pond culture of red-spotted grouper and red snapper (Doi et al., 1994a; Singhagraiwan et al., 1994). More recently in Australia the calanoid *Gladioferans imparipes* has been cultured (Payne & Rippingale, 2001) and used experimentally as a live food dhufish, pink snapper (Payne et al., 2001) and Western Australian seahorse (Payne, 2000).

High density, intensive cultures maintained under a rigorous set of environmental conditions require a significant level of input in terms of labour and biological resources. The advantages afforded by such culture systems lie in their controlled nature and the reliability of their live food output in terms of quantity and quality.

Of the three orders of copepods investigated for culture, there has been a bias toward the Calanoida and Harpacticoida, most probably as a consequence of the dominance of marine systems by the former (Huys & Boxshall, 1991) and the comparative ease with which harpacticoids may be cultured (Battaglia, 1970; Kahan et al., 1981; Zhang & Uhlig, 1993). The Cyclopoida have received less interest possibly as a result of their lesser representation in marine systems (Huys & Boxshall, 1991). However, this may be destined to change with recent recognition of the importance of marine cyclopoids, especially in the tropics, where they are represented in the smaller fraction of the zooplankton (Paffenhöfer, 1993; Hopcroft & Roff, 1996; Hopcroft et al., 1998).

1.5.1 Culture parameters

Despite the variety of culture systems developed by researchers conducting studies on specific aspects of copepod ecology or physiological responses, all need to be able to supply an environment suited to survival, reproduction and development. Two essential elements of system design lie in the maintenance of adequate water

quality facilitated by regular or continuous exchange of water, and the maintenance of an adequate food supply (Brownell & Horstman, 1987).

To maintain a healthy and reproductive population of an organism in an artificial environment, culture conditions must satisfy the physiological and physical requirements of the species. Food source, temperature and salinity appear to be the most critical components of the environment. The influence of light, volume, mixing and other parameters are of varying importance.

Fundamental to the success of marine copepod culture is the provision of high quality seawater. Crustaceans are sensitive to pollutants such as heavy metals and pesticides (Smith et al., 1994). Filtration to remove particulates and unwanted organisms is common, Zillioux & Lackie (1970) using a recirculating system incorporating a foam tower to reduce soluble protein and dissolved organic carbon loadings. Artificial seawater has also been used to provide uniform culture conditions (Barr, 1969; Heinle, 1969; Corkett, 1970).

Two factors are critical in determining growth rates of pelagic copepods: temperature and food supply (McKinnon, 1996), with temperature influencing the rate of development, and food concentration regulating egg production.

Salinities outside the optimal range require extensive adjustments to osmoregulatory mechanisms and affect the structural properties of aquatic invertebrates. Structural responses to salinity are based ultimately on differences in metabolism (Kinne, 1971) which affect relative growth of body parts and reproductive capacity (Milou, 1996).

1.5.1.1 Temperature and salinity

Despite the profound impact of food on the potential productivity of a copepod population through its relative ability to meet metabolic requirements for growth and development, temperature and salinity exert their influence on the rates at which growth and development occur (Hart, 1990).

Temperature and food abundance are the most important factors affecting the development rate (Uye, 1988; McKinnon, 1996) and body size (Klein Breteler & Gonzalez, 1988; Milou, 1996) of copepods. Every component of the metabolism of zooplankton is largely dependent on temperature (Heinle, 1969) with development time, fecundity and maturation time of egg sacs for several copepods being inversely related to temperature (Marshall, 1973; Allan, 1976; Hicks & Coull, 1983; Uye, 1988; Vijverberg, 1989; Milou & Moraïtou-Apostolopoulou, 1991a; Klein Breteler, 1994; Takahashi & Ohno, 1996). Huntley & Lopez (1992) found temperature alone explained more than 90% of variance in growth rates of more than 30 copepod species. Allan (1976) collated data on a variety of copepods and found low temperatures resulted in longer generation times and an increase in

body size, which in turn resulted in larger clutch sizes, an extension of embryonic development, and an increase in longevity.

Similarly, salinity can significantly affect the rates of growth, development in larval stages and reproduction of crustaceans, especially those with restricted capacity for osmoregulation (Kinne, 1971). Differences with response to salinity are also evident between geographically separated populations of the same species (Milou & Moraïtou-Apostolopoulou, 1991a; Lonsdale & Levinton, 1980). Hagiwara et al. (1995) observed a reduction in naupliar stage duration, increase in naupliar survival and longer life span at 32‰ than that recorded for *Tigriopus japonicus* at 4, 8 and 16‰.

The combined influence of temperature and salinity is most apparent in the production of dormant eggs by *Acartia californiensis*. Johnson (1980) observed that dormant eggs were produced below 15 °C irrespective of salinity, and while hatching of these dormant eggs was also temperature dependent, salinity was observed to regulate the rate and success of hatching.

The influences of temperature and salinity are apparent not only in developmental rates, but also in biochemical responses. Estuarine animals undergo large changes in the concentrations of numerous amino acids in response to the stimulus of osmotic stress. The upper limit for salinity tolerance is influenced by water temperature, the tolerance of salinity fluctuations decreasing as water temperature increases (Gonzalez & Bradley, 1994). Nanton & Castell (1999) demonstrated a similar effect of temperature on the essential fatty acid composition of harpacticoid copepod species *Tisbe* and *Amonardia*: *n*-3 HUFA decreased from 57% and 42% at 6 °C, to 47% and 35% at 20 °C, and 42% and 30% at 15 °C respectively.

In tropical ecosystems seasonal temperature effects are not as marked and may be largely ignored (Hopcroft et al., 1998). It is in these environments that salinity and food availability can exert dominant effects on copepod productivity.

1.5.1.2 Diet

All life cycle parameters are affected to a greater or lesser degree by the type of food available to copepods (Nassonge, 1970; Zurlini et al., 1978; Milou & Moraïtou-Apostolopoulou, 1991b; Swadling & Marcus, 1994). Important aspects pertinent to the success of a diet relate to the dominant food type present, the chemical composition of the food, and its abundance. In addition Rieper (1982) also alludes to the importance of the ability of the food to act as an appropriate substratum for bacteria to proliferate, a concept supported by Marshall (1973), Brown & Sibert (1977) and Hicks & Coull (1983) to mention a few.

Food is of fundamental importance to the survival of any faunal population. The suitability of a food source is determined by a combination of particle size, abundance and quality (Provasoli et al., 1970). However, it is the quality of the

food types falling within a suitable size range which ultimately determines the success of a diet in facilitating perpetuation of a population through its impact on reproduction.

Food quantity and quality

The most suitable diet is ultimately determined by the anatomical structure of the feeding appendages of the copepod being cultured (Anraku & Omori, 1963; Vanden Berghe & Bergmans, 1981). The quantity and quality of food presented to cultured copepods has a major impact on the success of the population. Significantly, mixed diets are superior to diets comprising single components (Klein Breteler et al., 1990).

It is evident that diets comprising a single component, although facilitating subsistence in many cases, often fail to maintain healthy reproducing populations of copepods. Numerous researchers have concluded that in order to achieve optimal productivity, mixed diets are required to provide a balanced diet containing all essential growth factors, vitamins and minerals, and present a food particle size range suited to all ontogenetic stages (Nassonge, 1970; Provasoli et al., 1970; Sellner, 1976; Paffenhöfer & Harris, 1970; Zurlini, 1978; Lee et al., 1985; Milou & Moraïtou-Apostolopoulou, 1991b; Ianora et al., 1995).

The efficacy of a food type depends on its digestibility and ability to fulfil the nutritional requirements of the species concerned, with food quality shown to be particularly important to crustaceans, especially with regard to fertility and fecundity (Provasoli et al., 1970; Milou & Moraïtou-Apostolopoulou, 1991b; Kleppel & Burkart, 1995). Dietary history and, more specifically, total lipid content of the diet influences the number of eggs produced (Gatten et al., 1980; Støttrup & Jensen, 1990). Food levels also affect the number of eggs per brood and the amount of growth per moult (Vijverberg, 1989). The low productivity occasionally observed in culture because of low egg production may be due in part to low food concentrations (Støttrup & Jensen, 1990). Zurlini et al. (1978) found a good correlation between algal suspension and egg production, maximum life span being associated with an intermediate concentration of food approximating $6x10^3$ cells mL^{-1}.

Zurlini et al. (1978) observed a considerable increase in life span and number of egg sacs produced per female as algal concentrations increased from $6x10^2$ to $6x10^3$ cells mL^{-1}. Above $6x10^3$ cells mL^{-1} little extension of adult life span was induced, but the number of egg sacs and eggs produced per female increased as food concentration rose from $6x10^3$ to $6x10^5$ cells mL^{-1}. In addition, the mean interval between egg sac deposition shortened from five days at $6 x10^3$ cells mL^{-1} to two days at a food concentration of $6x10^5$ cells mL^{-1}. This supported the findings of Corkett & McLaren (1969) regarding the influence of food concentration on egg production by *Pseudocalanus minutus*, where a reduction in

food supply resulted in an extension of the period between hatching of eggs and deposition of the subsequent egg sac. At maximum food concentration, the average time between laying of eggs was found to coincide with embryonic development time (Zurlini et al., 1978).

The rate at which food is consumed by copepods is influenced by particle size, abundance and quality with the relationship between ingestion and particle concentration described as nearly linear (Støttrup & Jensen, 1990). However Paffenhöfer & Van Sant (1985) observed saturation levels and minimum threshold levels below which ingestion ceased completely.

Nassonge (1970) reported that *Euterpina acutifrons* nauplii were unable to eat particles larger than 16 µm, the adults unable to utilise particles smaller than 7 µm. In general the optimum and maximum particle size increases as the individual copepod develops to maturity (Fernández, 1979; Berggreen et al., 1988; Tóth & Kato, 1996). The size (usually diameter) of particles filtered by copepods lies between 5 and 50 µm, with particles exceeding 30 to 50 µm being seized raptorially (Marshall & Orr, 1956; Marshall, 1973; Allan, 1978; Brown & Sibert, 1977). The ingestion of bacteria and particulate matter of dimensions less than 5 µm is achieved through scraping of surfaces by maxillipeds (Decho & Fleeger, 1988).

At high concentrations of algal cells, the reduced clearance rate observed may be related to gut passage time, algal size and relative retention abilities. A valid lower food size limit of algae for all copepods would appear to be in the range 2 to 4 µm, although *Calanus pacificus* exhibits a higher limit in the range of 4 to 11 µm, with a progressive increase in size from nauplii through to adult (Fernández, 1979; Berggreen et al., 1988; Støttrup & Jensen, 1990). This may imply that pelagic bacteria do not constitute a major dietary ingredient of suspension feeders and it is more likely that the detrital-grazing harpacticoids form a more direct link between the microbial loop and the classical food chain (Berggreen et al., 1988; Støttrup & Jensen, 1990). More recently work conducted in tropical waters has identified naupliar consumption of picoplankton as a significant link between the microbiological food chain and traditional food webs (Roff et al., 1995; Hopcroft & Roff, 1998). Picoplankton accounts for up to 30% of available food not able to be utilised by copepodid stages (Hopcroft & Roff, 1998).

The observed rate of ingestion does not appear to reflect nutritive value as evidenced by continued consumption of bacterial cells resulting in the extinction of the copepod population (Guidi, 1978). Copepods have also been observed to ingest high and low nitrogen content *Gracillaria* sp. at comparable rates (Guidi, 1984), and bacterial strains which did not result in copepod egg production (Rieper, 1982; Milou & Moraïtou-Apostolopoulou, 1991).

Feeding Biology

Growth and reproduction are a function of the morphology and behaviour of the organism, the concentration of food, food quality and the suitability of the physical environment (Vijverberg, 1989). Raptorial and filtering modes represent the major feeding mechanisms employed by copepods with the latter predominant among the pelagic zooplanktonic forms (Allan, 1976; Vijverberg, 1989). Despite uniformity in the functional anatomy of free-living copepods (Russel-Hunter, 1979), there are different mechanisms of feeding between the three orders.

Feeding preferences in copepods appear to be correlated with the morphological structure of the mouthparts. Comparison of the feeding preferences of six copepods offered either the diatom *Thalassiosira fluviatilis,* or *Artemia* nauplii, or a mixture of both revealed that the preference for a particular food was correlated with the morphological structure of the mouthparts (Anraku & Omori, 1963; Brown & Sibert, 1977; Vanden Berghe & Bergmans, 1981). In herbivorous species, such as *Calanus finmarchicus*, the second antennae with long plumose setae, mandibular palps, first maxillae and maxillipeds are well developed to collect suspended particles. In predatory species, such as *Tortanus discaudatus*, the mouthparts have few setae and are on the whole much simpler, the first and second maxillae and maxillipeds are modified as prehensile appendages, and the cutting edges of the mandibles exhibit very sharp, strong teeth. Omnivores, such as *Acartia tonsa*, possess appendages with structures intermediate between those of the grazers and predators, with the second maxillae employed in part for filtering and partly for grasping. The teeth of omnivores are generally heavier than those of herbivores, but not as stout as those possessed by carnivores. The three feeding groups follow a general decrease in setation and increase in structure strength with a progression from a herbivorous to carnivorous feeding habit. The survival of a copepod within any particular environment is therefore primarily dependent upon the morphology of its feeding appendages and availability of suitable food.

Food selection processes observed among copepods are influenced by the size, shape, availability and chemical composition of the specific food particle. Most investigations of selectivity have involved the use of microalgae (Richman & Rogers, 1969; Nassonge, 1970; Poulet & Marsot, 1978; Huntley, 1982; Price & Paffenhöfer, 1984; Paffenhöfer & Van Sant, 1985; Cowles et al., 1988) with others looking at preferences for other food types such as bacteria, protozoa, a variety of organic sources (Rieper & Flotrow, 1981; Rieper, 1982) and inert materials (Paffenhöfer & Van Sant, 1985). Each copepod species reacts specifically towards a given food substrate, depending on its chemoreceptive abilities (Poulet & Marsot, 1978; Guerin & Rieper-Kirchner, 1991). Some species of copepod are capable of discriminating between different bacterial strains (Rieper, 1982), and also between bacteria and other trophic sources (Guérin & Rieper-Kirchner, 1991).

There are ontogenetic shifts in the mode of feeding in the harpacticoid copepod *Nitocra lacustris*; nauplii scrape diatoms removing their mucilage and associated bacteria whereas copepodids actually ingest the diatom and associated bacteria whole (Decho & Fleeger, 1988). More recently Roff et al. (1995) determined that the nauplii of tropical copepod species represent an important link between the microbial food chain and the traditional food web consuming significant amounts of bacteria and picoplankton.

Discrimination between particles of similar size on the basis of chemical composition is the result of apparent perception of the cells in the fluid prior to contact with feeding appendages and detection of dissolved chemicals in the water (Poulet & Marsot, 1978; Cowles & Strickler 1983; Price & Paffenhöfer, 1984). Particular dinoflagellates produce a decrease in ingestion rates. Those exhibiting bioluminescence being ingested at significantly reduced levels in comparison to non-bioluminescent strains of the same species (Huntley et al., 1986).

The presence of both mechanoreceptors and chemoreceptors on the first antennae of copepods have been implicated in the perception of prey, with the possibility of both pre-capture and post-capture discrimination occurring (Cowles & Strickler, 1983; Paffenhöfer & Van Sant, 1985). Hammer & Brockman (1983) demonstrated that the excretion of six amino acids by the diatom *Thalassiosira rotula* was coupled to the physiological activity of the microalgal cells, and increased during periods of increased photosynthetic activity. As amino acids are perhaps the most important phagostimulant of crustacean chemoreceptors (Cowles et al., 1988), it is reasonable to assume amino acids are an important factor in food selection by copepods.

Clearance and ingestion rates have been shown to be a function of long range olfaction and short-range chemoreception (Paffenhöfer & Van Sant, 1985). The role of diffuse chemical stimuli as opposed to microzone or near cell chemical stimuli has been confirmed by the higher ingestion rates in the presence of algal exudate from faster growing cells (Cowles et al., 1988). Signal strengths, and therefore rates of capture and feeding, could be a function of cell size or nutritional content, and should prevent ingestion by a copepod of food items of little or no nutritional value. This theory is supported by the observation that non-viable organic particles, followed by faecal pellets, are ingested at slower rates than living particles (Paffenhöfer & Van Sant, 1985). Particles are not rejected simply because they are of little value as food, as copepods will ingest and form faecal pellets from indigestible materials such as Indian ink (Marshall & Orr, 1952) or polystyrene beads (Paffenhöfer & Van Sant, 1985).

1.5.1.3 Other considerations

Light in terms of photoperiod and spectral composition can influence copepod development (Milou, 1992) and behaviour (Davis, 1984). Diurnal movements

through the water column are related to light levels which in turn also affect the relative nutritive value of phytoplankton. Under artificial conditions, Milou (1992) found continuous illumination of *Tisbe holothuriae* cultures delayed the appearance of the first egg sac and induced high mortality of nauplii prior to hatching. Optimal photoperiod and spectral composition is known to stimulate development rates and results in shorter body length (Milou, 1992) coincident with optimal rates of growth, maturation and reproduction. In contrast, continuous illumination and long-day photoperiod also significantly reduced naupliar survival and the proportion of females developing within a population (Milou, 1993). At the other end of the spectrum, complete darkness entirely suppressed hatching of *Acartia clausi* eggs (Landry, 1975).

Similarly, dissolved oxygen levels less than 0.02 mL O_2 L^{-1} entirely suppressed hatching of four temperate water calanoid species (Lutz et al., 1992). The period of anoxia affected development times when eggs were re-exposed to oxygenated waters in which dissolved oxygen levels were greater than 0.15 mL O_2 L^{-1}.

The provision of appropriate light and aeration levels can be further improved on by the regular exchange of culture media. Zhang & Uhlig (1993) found flow-through systems to be associated with reduced naupliar mortality, reduced naupliar development time, and high naupliar production.

Fava & Crotti (1979) also observed that the daily renewal of water diminishes the effects of overcrowding, most probably as a result of dilution or removal of a 'non-specific' simple chemical compound which is implicated in the regulation of a constant population density. The greater proportion of females observed at lower population densities in flow-through systems was coincident with reduced pH fluctuations and lower levels of chemical compounds (Zhang & Uhlig, 1993).

1.5.1.4 Volume

Culture volumes are usually dictated by the topic under investigation (Paffenhöfer & Harris, 1979). It is also recognised that no single volume may be appropriate for the efficient culture of a species through all of its developmental stages (Paffenhöfer & Harris, 1979).

Volume greatly influences the viability of cultures, behaviour and offspring reproduction (Provasoli et al., 1970). Small volumes limit the ability to manipulate factors effectively. In addition there are difficulties with respect to maintaining food levels and countering the effects of accelerated evaporation (Barr, 1969).

The validity of any scientific investigation lies in the reproducibility of results and the level of replication employed in the trials. Smaller culture volumes present the advantages of requiring fewer individuals when stocking and are easily replicated. However, the results obtained in small volume cultures are not always directly transferable to larger scale systems which benefit from a greater flexibility with

respect to their ability to absorb, and therefore buffer, the effects of fluctuations in pH, nitrogenous waste products, dissolved oxygen levels and temperature. With these considerations in mind it is possible to obtain indicative information in small-scale trials which may be used to identify optimal ranges, the details of which may be tested on a larger more useful scale without 'wasting' resources.

1.5.2 Culture systems

A variety of culture systems ranging from small volume individual cultures to extensive cultures covering hectares have evolved in response to the various environmental and physical parameters which need to be assessed. In the field many factors act simultaneously but the importance of these factors can be determined in laboratory-based experiments where one factor can be varied whilst the others remain constant under controlled conditions (Milou, 1993). Laboratory culture has proven to be the most reliable means to accurately estimate growth and development times of zooplankton organisms (Hicks & Coull; 1983; Webb & Parsons, 1988; Vijverberg, 1989).

The effects of temperature, salinity, pH, light, oxygen concentration, food supply (both quantity and quality) and zooplankton density have been examined by means of cultures maintained under controlled environmental conditions in the laboratory (Nassonge, 1970; Sellner, 1976; Zurlini et al., 1978; Fernández, 1979; Kahan, 1979; Palmer & Coull, 1980; Rieper & Flotow, 1981; Vanden Berghe & Bergmans, 1981; Guidi, 1984; Lee et al., 1985; Støttrup & Jensen, 1990; Guerin & Rieper-Kirchner, 1991; Milou & Moraïtou-Apostolopoulou 1991; Klein Breteler & Schogt, 1994). The primary motivation for such work is the interpretation of field data (Milou, 1996) including a greater insight into factors affecting juvenile recruitment to wild fish stocks (Kiørboe et al., 1985; Chang & Lei, 1993; Poulet et al., 1995).

Culture systems may be categorised as one of four types: batch, recirculating, flow-through and *in situ*. These will be addressed in the following sections.

1.5.2.1 Batch culture systems

Batch culture methods refer to systems to which are added a population of zooplankton, subsequently maintained and on-grown to produce a final population harvested in its entirety. Batch culture methods are the most common means used to maintain both marine and freshwater zooplankton populations (Vijverberg, 1989). This technique is limited by inherent problems related to the decrease in food cell concentration, associated with the grazing and settling of algae and also the accumulation of excretion and egestion products with time. Together with the accompanying pH and oxygen level changes, such changes to the culture media are inhibitory to growth through adverse effects on feeding (Gerritsen & Porter, 1982).

The observed increase in food density at, and just above, the bottom of culture vessels may result in the development of undesirable effects due to fouling caused by the accumulation of bacterial metabolites and products of decomposition. Such adverse effects may be largely avoided through the

- frequent renewal of the culture medium which facilitates maintenance of oxygen levels and removal of metabolic wastes (Barr, 1969; Vijverberg, 1989),
- maintenance of zooplankton density or biomass at a constant low level reducing the source of organic wastes (Fava & Crotti, 1979);
- reduction of nutrient loading by limiting the food concentration to or below the incipient limiting concentration (Kahan et al., 1982; Vijverberg, 1989);
- use of mobile flagellate microalgae as food (Paffenhöfer & Harris, 1979; Vijverberg, 1989);
- use of axenic culture systems (Vijverberg, 1989).

The inclusion of a substrate, generally comprising autoclaved material from the original habitat of the animal, assists the culture of some benthic copepods (Palmer & Coull, 1980; Alongi, 1985; Chandler, 1986).

A variety of batch culture systems have evolved to provide information on the organism in culture. Two primary categories of batch culture systems are the individual batch culture systems, corresponding to a single individual being maintained in a small culture, and the mass batch culture systems entailing the maintenance of a population of organisms in a large culture volume.

Individual batch cultures provide a means by which to follow the development of a single individual over time and have proven extremely popular in the determination of growth and development rates, and the effect of various environmental parameters on productivity and nutritional value of zooplankton organisms (Anraku & Omori, 1963; Sellner, 1976; Zurlini et al., 1978; Fava & Crotti, 1979; Kahan, 1979; Vanden Berghe & Bergmans, 1981; Guidi, 1984; Guerin & Rieper-Kirchner, 1991; Zhang & Uhlig, 1991). There is a range of designs for individual cultures ranging from simple culture tubes comprising 100 mL glass test tubes sealed with cotton wool and suspended in a water bath (Vijverberg, 1989), through to Lonsdale & Levinton's (1980) multi-depression dishes, to the more complex thermal gradient incubator of Thomas et al. (1963) who made use of a single incubator capable of maintaining eight different temperatures.

Mass cultures generally use larger volumes of growth media than individual cultures, primarily to compensate for the larger number of individuals contained by the system. The volume available to each organism varies from 2.5 mL up to 500 mL per animal (Vijverberg, 1989). Within these culture systems the density

generally increases with time associated with a gradual decrease in volume per animal. Under mass culture conditions the larger number of individuals per unit surface area and greater detrital loading due to excretion and egestion products highlights the disadvantages of static culture. Modification of culture systems to address these aspects have resulted in the development of stirred and aerated cultures.

Stirred cultures involve the use of mechanical stirring, rotation or aeration to maintain a suspending water current. The cumbersome nature of the more mechanical stirring apparatus such as rotating vessels and Greave's plankton Kreisel has limited their use, in addition to the potential damaging effects on the copepods themselves as a direct result of the presence of stirring equipment in the culture vessel (Vijverberg, 1989).

Aerated mass culture is most often employed for marine calanoids utilising large 10 L to 1000 L vessels into which compressed air is blown into the medium at the tank bottom. The concept of mass aerated culture is employed in the aquaculture industry in the rearing of live foods such as the rotifer *Brachionus plicatilis* and the brine shrimp *Artemia*. Støttrup et al. (1986) modified small scale production to supply 250 000 nauplii of the calanoid copepod *Acartia* per day using a combination of 200 L PVC tanks demonstrating the possibility of continuous harvest from a batch culture system

1.5.2.2 Recirculating systems

Recirculating systems involve the recycling of the culture medium within the system with water quality enhanced by the use of mechanical and/or biological filtration. Partial water exchanges may be made to facilitate the addition of food or the replenishment of culture media volume lost with the harvest of a portion of the zooplankton population.

Sun & Fleeger (1995) developed a sustainable mass culture system for the harpacticoid *Amphiascoides atopus* comprising culture tanks, collectors, filters and aerated reservoir from which the culture water was recirculated. The 4 m^2 basal surface area of the culture tanks was capable of producing in excess of 1 million individuals and over 5 grams wet biomass per day when fed *Chaetocerous muelleri* and commercial fish flakes.

In Western Australia an intensive culture system for the calanoid *Gladioferans imparipes* comprising 500 L culture vessels fitted with biological filtration and protein skimmer provided with unicellular algae on a daily basis produced in the order of 500 000 nauplii (Payne & Rippingale, 2001).

1.5.2.3 Flow-through continuous culture systems

Flow-through systems constitute some of the most technically advanced systems and involve the continuous replenishment of the medium containing food. The constant flow of the medium containing food reduces problems of settling and aids in the removal of bacteria and dissolved waste products such as ammonia and other contaminants (Vijverberg, 1989). The most complex of these culture systems comprise a chemostat for culturing algae, a dilution unit with membrane-filtered water from the natural habitat of the animals, and a culture vessel for the experimental animals. The algal suspension flows continuously from the chemostat and is mixed with the filtered water and pumped into the culture vessel. Although a very appealing system for measuring growth and development rates under conditions not limited by food deprivation, variation in water chemistry or build up of metabolites, factors such as complexity and cost, and the potential for contamination, have negated widespread use of such systems in laboratory-based studies. However such systems have proven beneficial to the culture of copepods for use in aquaculture (Urup, 1994; Støttrup & Norsker, 1997).

Despite being classified as a batch culture system by Vijverberg (1989), floating culture tubes are more representative of a flow-through system. The sand-dwelling harpacticoid *Asellopsis* sp. has been successfully cultured in floating tubes maintained in a large temperature controlled tank of seawater (Hardy, 1978) with both ends fitted with gauze netting. The animals were thus contained but free flow of water permitted exchange with the larger volume of seawater. Aeration of the large tank agitated surface water with the subsequent up and down motion of the tubes causing the basal net to flex slightly, thus improving water movement across the gauze.

Aquaculture application may be found in the floating net-type baskets used by Kahan et al. (1982) who demonstrated that benthic *Tisbe* and *Asellopsis* species could be cultured in the restricted upper layer of the water column. Such techniques have been promoted as of potential commercial use in the supply of naupliar feed to fish larvae present in the underlying tank. However, the use of such floating basket cultures is not practical due to the subsequently reduced light levels and water quality in the larval rearing tanks.

1.5.2.3 In situ culture systems

In situ culture systems aim to emulate the natural physical environment and ecology of the organism under scrutiny having evolved from a desire to investigate zooplankton population dynamics under conditions resembling as closely as possible the natural environment (Vijverberg, 1989; Swadling & Marcus, 1994). The culture techniques include both open- and closed-systems and are most often combined with cohort analysis methodology. Open systems involve the selective restriction of zooplankton organisms allowing free movement of various food

articles transported in the water flowing through the system. Fouling of such systems may influence through flow and food exchange rates. Conversely, closed systems prevent through flow from the external environment. The comparatively higher rates of zooplankton production associated with such systems may be attributable to the accumulation of phytoplankton and detritus, promoting growth of bacteria and ciliates. The subsequent microcosm of the enclosure thus exhibits better food conditions than the external situation.

Urup (1994) described the use of natural zooplankton harvest in open systems such as fjords. Larvae were released into an isolated volume of water or zooplankton was harvested by the filtration of large volumes of water and concentrated into polythene bags into which the larvae are released. These systems are cheap to establish and maintain and allow the exclusion of predators through either filtration or chemical treatment. An alternative to these semi-extensive systems are more closely controlled intensive systems.

The ultimate intensive *in situ* copepod culture system is represented by that used in the production of turbot in Denmark; the Maximus company computer-supported subjective decision manipulating program which regulates environment parameters to maximise copepod production for live food (Urup, 1994). Calanoid copepods are held in large tanks representative of a natural ecosystem maintained under laboratory culture conditions. The system is maintained in a steady state by a Computer Supported Subjective Decision Manipulation Program (CSSDMP) which matches phytoplankton production with consumption by copepods, and predation of copepods by fish. System productivity is optimised as a result of complex modelling and feedback systems interpreted by the CSSDMP. The model is constantly being adjusted to incorporate new elements such as the characteristics of new algae and varying levels of production output (Urup, 1994).

The system chosen by an aquaculture facility is largely dependent on the available resources.

1.5.3 Measures of culturability

The effects of various environmental parameters on fecundity, mortality and longevity of individuals, exerts a significant influence on population dynamics. The effect of various environmental factors on the individual is amplified when the population is considered as a whole.

Different environmental parameters can affect different aspects of demography independently, and in opposing directions, often with conflicting results on productivity when considered in isolation (Zurlini et al., 1978). The value of the demographic descriptors T, R_o, and r_m lies in the ability to compare the performance of individuals maintained under different culture conditions.

Demographic variables reflect the potential for the maintenance of a population in a given environment. The calculation of these factors has the advantage of combining the effects of many physiological processes, which if considered separately would yield different results (Allan, 1976; Zurlini et al., 1978; Milou & Moraïtou-Apostolopoulou, 1991 a,b). Bergmans (1984) expressed concern at the bias of demographic parameters when estimating absolute productivity of aquatic systems. However such bias was considered to be acceptable for certain purposes such as determining the type of influence a particular environmental parameter may exert (Bergmans, 1984) as it relates to the comparison of culture conditions for live food species.

Three demographic descriptors of importance are net reproduction rate, intrinsic rate of natural increase and mean generation time, the components of which may be influenced by a number of interactive factors. The influence of various environmental factors may also be determined by monitoring relative increases in population density and the distribution of individuals between demographic groups over time.

1.5.3.1 Net reproductive rate (R_0)

The net reproductive rate (R_0), the number of times a population will multiply per generation, is determined by the replacement rate of females from one generation to the next within the population.

Zurlini et al. (1978) defined R_0 as the sum of the product of the percentage of females surviving at the beginning of each age interval and the average number of female offspring produced in each age interval by a female of a given age:

$R_0 = \Sigma\, l_x.m_x$ Zurlini et al. (1978)

where l_x is the fraction of females surviving at the beginning of each age interval, and m_x the mean number of female offspring produced in each age interval by a female aged x.

Guérin & Rieper-Kirchner (1991) and Milou & Moraïtou-Apostolopoulou (1991 a,b) defined R_0 as the ratio of the number of third generation females to second generation females, with the number of G3 females dependent on the total number of G2 offspring, the mortality during larval development and the sex ratio of the adults:

R_0= G3/G2 (Milou & Moraïtou-Apostolopoulou, 1991 a,b)

where G3 is the number of female offspring developing in the third generation, and G2 is the number of female offspring developing in the preceding second generation.

1.5.3.2 Intrinsic rate of natural increase (r_m)

The intrinsic rate of natural increase is characteristic of a population's ability to proliferate. The intrinsic factors known to affect r_m are principally fecundity, longevity and rate of development, with temperature, nutrition and salinity comprising the major extrinsic factors (Hicks & Coull, 1983). Reproductive potential usually increases in optimal, as opposed to suboptimal food regimes. Similarly, because generation times decrease with increases in temperature toward the optimum, optimal temperature also results in elevated r_m (Hicks & Coull, 1983).

The methods applied to calculate r_m both use information pertaining to the female portion of the population only, and are thus affected by the survival rate of females and the sex ratio of the population:

$\Sigma e^{-r_m x} . l_x . m_x = 1$ (Zurlini et al., 1978)

where l_x is the fraction of females surviving at the beginning of each age interval, and m_x the mean number of female offspring produced in each age interval by a female aged x.

$r_m = (\ln R_0)/ T$ (Milou & Moraïtou-Apostolopoulou, 1991a)

where R_0 is the calculated net rate of reproduction and T is the mean generation time.

Guérin & Rieper-Kirchner (1991) use the same terms as Milou & Moraïtou-Apostolopoulou (1991a, b) but refer to it as specific growth rate. The intrinsic rate of natural increase expresses a species' development potential per unit time and facilitates direct comparison between species exhibiting different life cycles.

The maximal rate of increase (r_{max}) represents the physiological capacity of the population of a given species to increase in a given environment (Allan, 1976). The productivity of any population is directly correlated with survival rates, sex ratio, number of eggs per female and development rate. An increase in any component results in greater productivity.

1.5.3.3 Mean generation time (T)

The mean generation time (T) corresponds to the average interval between the birth of an individual and the appearance of its progeny. Mean generation time is strongly influenced by environmental parameters through their influences on development time, maturation time of eggs and the time interval between the extrusion of two successive egg sacs by a single female.

Two methods are used to determine T: direct observation of individuals providing a temporal measure, and calculation based on characteristics of females and their resultant progeny.

Zurlini et al. (1978) defined T as the natural log of the ratio of net reproductive rate (R_o) and the intrinsic rate of natural increase (r_m):

$$T = \frac{\ln R_o}{r_m} \qquad \text{(Zurlini et al., 1978)}$$

Guérin & Rieper-Kirchner (1991) and Milou & Moraïtou-Apostolopoulou (1991 a,b) described T as the ratio of the sum of the number of nauplii hatching from the egg sac produced by the female multiplied by the age of the female at the time of hatching, and the sum of the number of nauplii hatching from the egg sac.

$$T = \frac{\sum x.Ux}{\sum Ux} \qquad \text{(Milou \& Moraïtou-Apostolopoulou, 1991 a,b)}$$

where x is the age (in days) of the female (G2) at the time of hatching of nauplii and Ux is the number of nauplii per egg sac of the female offspring.

These three demographic descriptors (net reproductive rate, intrinsic rate of natural increase and mean generation time) are considered to be important when comparing the relative growth rates achievable under different culture conditions (Allan, 1976; Milou & Moraïtou-Apostolopoulou, 1991a; Guérin & Rieper-Kirchner, 1991). They have been used widely to compare productivity of cultured populations maintained under different conditions for varying lengths of time and will be used in this study for similar comparisons.

1.5.3.4 Cohort analysis

The growth rate of zooplankton populations in the sea can be measured by following the development of the successive cohorts (Uye, 1988, Klein Breteler & Schogt, 1994). A cohort analysis is often difficult for populations which are characterised by continuous reproduction, short generation times and variable mortality, and is often predicted from the data obtained from rearing experiments conducted under controlled conditions (Uye, 1988).

Not all copepods exhibit isochronal development (Chang & Lei, 1993; Takahashi & Ohno, 1996; Doi et al., 1994b; Hopcroft & Roff, 1996) with some species exhibiting rapid growth through early naupliar stages, or delayed development in late naupliar or copepodid stages. Hart (1990) stated that the high frequency of non-isochronal development in copepods negates the validity of the fundamental assumption of equivalent duration of all life stages.

Cohort analysis, life cycle evaluation and elemental tracking have been further quantified and qualified through controlled experiments conducted in modified enclosures located in the natural environment (Kimmerer & McKinnon, 1987; Jónasdóttir, 1994). Studies have also been conducted under artificial conditions emulating those of the natural environment in an attempt to gain a finite understanding of the processes affecting copepod productivity and hence larval fish recruitment.

1.5.3.5 Egg production

Fecundity in the field has been used as an indicator of feeding conditions based on the fact adult copepods do not moult and that under steady conditions adult females express all their production not as somatic growth, but as egg mass (McLaren & Leonard, 1995). Copepod egg production is primarily related to food supply (McKinnon, 1996) and as such has been used as a means by which to determine the influence of natural plankton assemblages on copepod productivity (Rodríguez et al., 1995; Saiz et al., 1997), and as an indicator of the value of a food type to copepod culture (Arnott et al., 1986; Støttrup & Jensen, 1990). Debate regarding the suitability of this method relates to the fact that some algae support excellent copepod growth, but do not support egg production (Turner et al., 2001), and in the case of some diatoms actually adversely affect egg viability (Ianora et al., 1995; Uye, 1996; Turner et al., 2001). The indication is that responses may well be species specific, and care should be taken in extrapolating results between populations.

1.5.3.6 Life stage abundance

From an aquaculture perspective, the most useful demographic property is mean generation time which indicates the period of time during which the life stages of copepod species are available to fish larvae. Another useful measure of culture productivity is the fluctuation in the total number of individuals within a population over time under the prevailing maintenance regime.

Doi et al. (1994) and Singhagraiwan et al. (1994) assessing the impact of fertilisation levels on copepod production in 2.5 hectare earthen ponds relied on the use of total numbers of each copepod species within the zooplankton to assess the optimal regime.

The ability to recognise and track cohort development within the monitored populations enabled Doi et al. (1994b) to calculate stage-specific mortality. Where mass cultures of copepods are maintained, monitoring the total numbers provides the most convenient means of assessing the health and relative productivity of the culture.

1.6 Copepod taxa selected

The harpacticoid *Tisbe* sp and the cyclopoid *Apocyclops dengizicus* were found to persist in rotifer cultures maintained at aquaculture facilities suggesting their amenity to mass culture. The calanoid *Acartia* species was chosen because of its documented larval acceptance and proven requirement for the successful culture of lutjanid larvae (Singhagraiwan & Doi, 1993; Schipp et al., 1999).

Ideal characteristics of a copepod species suitable for culture include: (a) rapid turnover, (b) tolerance of a wide range of environmental conditions, (c) simple dietary requirements and (d) tolerance of handling.

These four characteristics of culturability in combination with the most important consideration, that of acceptance by larval finfish, constitute the five criteria determining the suitability of a species for mass culture as a live food.

1.6.1 *Tisbe* species

The Key Centre for Aquaculture at the University of Tasmania in Launceston commenced research into the hatchery production of greenback flounder in 1989 (Hart, 1994). The long-term goal was the development of a pilot scale commercial hatchery providing the basis for the future diversification of Tasmanian finfish aquaculture through the provision of an alternative finfish species to Atlantic salmon. The subsequent discovery that flounder may be latent carriers of atypical *Aeromonas salmonida* has meant that polyculture is no longer an option in Tasmania, however flounder is still an alternative mariculture species exhibiting potential in Victoria where there is no established aquaculture industry based on Atlantic salmon (Mozqueira, 2000).

Running concurrently with flounder research were trials on striped trumpeter as another alternative mariculture species. Unlike flounder, striped trumpeter larval survival was low and accompanied by the appearance of deformities, possibility attributable to dietary deficiencies (Cobcroft et al., 2001).

The successful production of 50,000 post-metamorphosic juvenile flounder was accompanied by an unacceptable level of both morphological and pigmentation abnormalities (John Purser, University of Tasmania, *pers. comm.*). These abnormalities were attributed to a combination of environmental and nutritional deficiencies.

Similar abnormalities, particularly mal-pigmentation, were experienced in the culture of halibut and turbot in the Northern Hemisphere. The main body of evidence suggests that nutritional essential fatty acid deficiencies and imbalances were the primary cause of mal-pigmentation (Kuhlmann et al., 1981; Witt et al., 1984). Overseas, the inclusion of copepods in the larval diet alleviated the high incidence of both morphological and pigmentation aberrations (Kuhlman et al.,

1981; Witt et al., 1984; Segner et al., 1993). The general improvement in survival, growth and normal development of turbot larvae was largely attributed to the nutritional profile of the copepods used in the larval rearing stages. The inclusion of copepods in the diet also resulted in an increased stress tolerance, and reduced mal-pigmentation found in turbot and halibut (Kuhlman et al., 1981; Kraul et al., 1992).

Harpacticoids are a natural component of wild marine fish larvae including those of sole, plaice, herring, seabream and sea bass (Norsker & Støttrup, 1994), and on tropical marine reefs, they represent an essential food source for juveniles of many demersal species (Alheit & Schiebel, 1982).

An extension of the Northern Hemisphere research is the use of southern temperate copepod species in the larval rearing of Southern Hemisphere flatfish. In view of the reported potential of *Tisbe* sp. as a live food organism (Kahan et al., 1981; Norsker & Støttrup, 1994), its cosmopolitan distribution (Williams & Jones, 1994), and its hardy nature under artificial culture conditions (Zhang & Uhlig, 1991), in combination with ease of collection, *Tisbe* is considered to be worth further investigation in Australia.

Flounder was used as a model marine fish given the relative ease with which larvae could be obtained, and the potential for copepods to be used in its culture in the future.

1.6.2 *Apocyclops dengizicus*

In contrast to *Tisbe* and *Acartia*, interest in *Apocyclops dengizicus* arose from inauspicious beginnings. Concerns were expressed by barramundi farmers that this cyclopoid was predating barramundi larvae introduced to their green-water ponds, and that it was this cyclopoid that was to blame for poor barramundi harvests.

Barramundi has been cultured in Australia since 1984, initially using methods developed in Asia (Battaglene & Fielder, 1997). The preferred larval rearing method used in the culture of barramundi in the Northern Territory has been extensive green-water culture reliant on the fertilisation of natural waters in earthen ponds. The natural zooplankton of these ponds provide the source for the rotifer *Brachionus rotundiformus* cultures used by the Darwin Aquaculture Centre (DAC). The subsequent rotifer cultures were inherently contaminated by copepodids at the time of collection, and with continued culture at the DAC the persistent copepod was found to be a cyclopoid. The polyculture of live foods was not considered a problem when rearing barramundi as the presence of the cyclopoid appeared to improve rotifer culture, and barramundi larvae were seen to ingest the various copepod stages collected with the rotifers during harvest (Schipp *pers comm.*).

Preliminary investigation into the composition of the zooplankton of green-water extensive barramundi rearing ponds revealed the presence of *Apocyclops*

dengizicus in small numbers (Appendix C). Despite this low natural abundance, *A. dengizicus* was considered as a candidate as a result of its continued presence in rotifer cultures at the DAC, its availability to barramundi in local rearing systems and the reported use of other *Apocyclops* species in tropical mariculture (James & Al-Khars, 1984; Chang & Lei, 1993; Su et al., 1997).

1.6.3 *Acartia* species

The Northern Territory aquaculture industry expanded into barramundi production in the early 1990s (Schipp, 1996). The low level of infrastructure use in the Northern Territory and close proximity of northern Australia to the lucrative South East Asian market (Rimmer et al., 1997) prompted the search for alternative finfish aquaculture species.

Golden snapper was selected by the Darwin Aquaculture Centre (DAC) in 1988 on the basis of its marketability as a firm white fleshed fish, prized as a commercial and recreational catch, and the reported success with lutjanid culture in Singapore and Thailand (Lim et al., 1985b; Singhagraiwan & Doi, 1993). Subsequent research on juveniles at DAC showed that the species could be reared at high densities, readily accepted pelleted feeds and tolerated a wide range of salinities (5 to 40‰) and attained 500 g in six months (Schipp & Pitney, 1995).

However, difficulties were experienced in the culture of golden snapper primarily as a result of unsuccessful digestion of live food at the commencement of exogenous feeding when larvae were presented with rotifers (Glenn Schipp, DAC, *pers. comm.*). Initially this was attributed to the inappropriate size and nutritional value of the rotifers offered. However the local rotifer proved to be a small strain rotifer *Brachionus rotundiformus,* and not *B. plicatilis* as initially thought. Enrichment of *B. rotundiformus* with various products also failed to improve golden snapper larval survival rates.

In 1996, the use of wild harvest zooplankton dominated by calanoid nauplii resulted in the first successful rearing of larval golden snapper. Work conducted on golden snapper in Singapore (Lim et al., 1985b), and on red snapper in Thailand (Singhagraiwan & Doi, 1993) suggested that it was the calanoid *Acartia* species component of the wild plankton that resulted in the success of lutjanid larval rearing. Lutjanid larvae were observed to start feeding on copepod nauplii, most probably *Acartia*, of 100-120 µm in length (Singhagraiwan & Doi, 1993). It was envisaged that a semi-intensive culture method might be suitable for the production of juvenile golden snapper for aquaculture.

Little information was available within Australia concerning the availability and culture of *Acartia* species. More specifically information was required for tropical *Acartia* species, as the majority of work conducted with members of the genus had been undertaken using temperate species.

1.7 Thesis outline and objectives

The primary objective of this thesis was to describe the culture parameters of three Australian copepod species; *Tisbe* sp., *Apocyclops dengizicus* and *Acartia* sp., identified by the author as potential live food candidates in the culture of marine finfish larvae.

The Australian aquaculture industry, in collaboration with the Australian Commonwealth Government, established a CRC for Aquaculture in 1993. The government matched funding provided by industry to address critical issues limiting the development of aquaculture in Australia. Six key areas were identified by the aquaculture industry as a high priority requiring immediate redress, one of which included the development of alternative foods for marine finfish larvae within the Nutrition Program.

The three copepod species chosen for this investigation represent three of the free-living orders of Copepoda; encompassing both tropical and temperate climates, and estuarine and marine species.

The scope of this thesis is broad; the work was conducted in view of applying the outcomes to the field of commercial aquaculture. The general aims of this study were threefold:

A. To document the life cycles of the three copepod species to provide basic information of value to the aquaculture technician. The first step toward the successful propagation of any organism is knowledge of the life cycle of the organism: the number of developmental stages, their duration and the tolerances exhibited by each developmental stage. The culture of copepods as a live food for marine fish larvae is no exception.

B. To obtain information on the culture characteristics of the three copepod species and to determine the limiting elements of culture systems including the influences of temperature, salinity and diet on culture productivity.

C. To use the three copepod species in feeding trials to assess their initial acceptance as a live food by cultured marine fish larvae.

These aims were achieved in the following manner:

Life cycle and demographics

Individual isolates of the three copepod species were monitored and the number of life stages, and their duration and dimensions were recorded. The information collected was assessed in light of the current body of literature available for Northern Hemisphere representatives of *Tisbe* sp., *Apocyclops dengizicus* and *Acartia* sp. to provide a concise summary of information of value to Australian aquaculture technicians.

Copepod culture

The relative influences of culture temperature, salinity and diet composition were assessed for the three copepods in terms of the population density and demographic composition. Environmental conditions which produced an optimum population density were identified.

Feeding trials with test finfish species

My research initially commenced in Tasmania with an investigation of the relationship between the temperate harpacticoid *Tisbe* sp. and greenback flounder. Husbandry trials with flounder at the University of Tasmania's Key Centre for Aquaculture were successful in obtaining juvenile flounder, however mal-pigmentation and deformities were unacceptably high at the time (John Purser, University of Tasmania, *pers. comm.*). It was thought that the inclusion of copepods in the larval diet might alleviate the problem as they had for turbot in the Northern Hemisphere (Kuhlmann et al., 1981; Witt et al., 1984; Urup, 1994). It was unknown whether flounder larvae would ingest life stages of *Tisbe* sp. or how the copepod would perform in a mariculture environment.

During my second year of research, collaboration fostered within the CRC for Aquaculture recognised the potentially greater need for research into copepod culture in the development of culture techniques for golden snapper as an alternative aquaculture species in the Northern Territory. In contrast to flounder, golden snapper larval survival on traditional live foods was essentially zero and was also the subject of research within the CRC for Aquaculture. Initial fact-finding conducted by Glenn Schipp of the DAC had determined the importance of copepods to the successful rearing of golden snapper, and had identified potential sources of *Acartia* species.

While conducting my research on *Acartia* species in Darwin, liaison with barramundi farmers supportive of the diversification of aquaculture in the Northern Territory raised the issue of the potentially predatory nature of a cyclopoid common in their larval rearing ponds. The copepod they identified as '*Apocyclops dengizicus*' exhibited traits desirable in a live food organism. Subsequently trials were conducted to determine the nature of the interaction between the cyclopoid and barramundi larvae.

The contents of the appendices provide background information supporting the work reported in the main body of the text. A number of investigations were undertaken during my candidature which, although important to the development of techniques and experimental design, do not constitute core results. Appendix B, corresponding to Chapter 3 *Apocyclops dengizicus* providing information on the zooplankton communities developing in semi-extensive aquaculture ponds in the Darwin region, is a clear example of an investigation important in terms of the background information provided, but not essential to the development of culture

techniques and the assessment of the suitability of copepods as a live food for larviculture of marine finfish species.

Chapter 2
Tisbe species

2.1 Introduction

The genus *Tisbe* belongs within one of the forty-seven families of Harpacticoida which are primarily benthic and inhabit both fresh and marine waters (Huys & Boxshall, 1991). *Tisbe*, which occur worldwide predominately in shallow marine waters, is one of 25 genera within the family Tisbidae, and encompasses about 60 described species (Dahms & Schminke, 1995). The actual number of species is unknown due to the difficulty in distinguishing between sibling species, and the lack of morphological information concerning Southern Hemisphere representatives within the genus.

Cross-breeding experiments conducted in the Northern Hemisphere have shown that species formally believed to be cosmopolitan, eurythermic and euryhaline are actually groups of morphologically similar sibling species (Volkmann-Rocco, 1971; Dahms & Schminke, 1995). Much debate exists as to the number of biological species within the genus *Tisbe*, the only true test for species being intersterility or genetic dissimilarity (Volkmann-Rocco, 1971). Dahms & Schminke (1995) completed a comparison of six species of *Tisbe* at both the morphological and the molecular level: *T. battagliai*, *T. bulbisetosa*, *T. cucumariae*, *T. furcata*, *T. gracilis* and *T. holothuriae*. It was deduced that absolute species identification required the use of molecular markers.

Table 2.1.1: The taxonomic hierarchy for the genus *Tisbe* (from Bowman & Abele, 1982; Huys & Boxshall, 1991).

Level	Title	
Subclass	Copepoda	Milne-Edwards, 1840
Superorder	Podoplea	Giesbrecht, 1882
Order	Harpacticoida	Sars, 1903
Family	Tisbidae	Stebbing, 1910

In view of the reported potential of *Tisbe* species as a live food organism for larviculture in the Northern Hemisphere (Kahan et al., 1981; Norsker & Støttrup, 1994), its cosmopolitan distribution (Vanden Berghe & Bergmans, 1981; Williams & Jones, 1994), and its hardy nature under artificial culture conditions (Battaglia, 1970; Ikeda, 1973; Zhang & Uhlig, 1991), in combination with ease of collection (Milou & Moraïtou-Apostolopoulou, 1991), it was considered to be a suitable candidate for further investigation in Australia.

2.1.1 Background information for *Tisbe* species

Tisbe holothuriae has been cultured in the laboratory since 1963 when it was used to keep cultures of folliculinids (ciliates) and small invertebrates (hydrozoans etc.)

clear of overgrowing microorganisms such as bacteria or fungi (Zhang & Uhlig, 1993). As early as 1965, Barr (1969) was able to maintain populations of *T. furcata* in 150 mL Griffin beakers for 1.5 years.

Since then a significant amount of research have been conducted with Northern Hemisphere congeners of *Tisbe* to quantify trophic transfers (Webb & Parsons, 1988; Milou & Moraïtou-Apostolopoulou, 1991c), the influences of environmental parameters and toxins on population dynamics (Fava & Crotti, 1979; Guérin & Kerambrun, 1982; Lee et al., 1985; Guérin & Rieper-Kirchner, 1991; Milou & Moraïtou-Apostolopoulou, 1991 a,b; Milou, 1993; Smith et al., 1994; Williams & Jones, 1994; Milou, 1996; Abu-Rezq et al., 1997; Sibly et al., 2000), genetic and evolutionary studies (Battaglia, 1970; Dahms & Schminke, 1995) and more recently as a live food for larviculture (Zhang & Uhlig, 1993; Norsker & Støttrup, 1994).

The research conducted has identified a number of important elements which affect the growth and productivity of *Tisbe* species populations. The influences of temperature, salinity, diet and to a lesser extent light, have been investigated.

The effects of temperature and food concentration on copepod development appeared to act independently on *Tisbe battagliai* (Williams & Jones, 1994). Copepods were able to survive on *Isochrysis galbana* provided at carbon concentrations of 1300 and 3250 µgC L^{-1}. Increased mortality was observed at the lower concentrations of 83, 203 and 520 µgC L^{-1} which was exacerbated by increasing the temperature from 15 to 25 °C.

Low temperatures between 14 °C and 18 °C were associated with higher lipid content of *Tisbe holothuriae* when food was present in excess (Milou & Moraïtou-Apostolopoulou, 1991c). Nanton & Castell (1999) were able to determine that the PUFA content, in particular AA, EPA and DHA of *Tisbe* sp. cultured at 6 °C, 15 °C and 20 °C decreased in the following order 6 > 20 > 15. Supporting the conclusion of Milou & Moraïtou-Apostolopoulou (1991c) that the use of medium temperatures (between 18 °C and 22 °C) produced the greatest number of nutritionally replete copepods for use as live food for use in larviculture.

Guérin & Kerambrun (1982) and Zhang & Uhlig (1993) also found 20 °C to be the optimal culture temperature, however the associated optimum culture salinities identified were quite different, 38 ‰ as compared to 28 ‰ respectively. Salinity influences growth rates and population dynamics indicate an optimal range as identified for temperature. Variations observed between cultured species may be explained by geographic separation of the species strains used (Milou, 1993). An important influence of salinity relates to its influence on copepod nitrogen content. An increase in the pool of free amino acids was observed when salinity increased from 26 ‰ to 38 ‰ (Milou & Moraïtou-Apostolopoulou, 1991c). Female body

size had also been recorded as increasing slightly with change in salinity from 26 to 44 ‰ (Milou, 1996).

Milou (1993) investigated the effects of two modalities of light, photoperiod and spectral composition, on the survival and sex ratio of *Tisbe holothuriae*. Mortality was found to increase from continuous lighting with the entire visible spectrum to constant darkness, and from red to blue wavelengths of illumination. Photoperiod influenced sex ratio with the relative number of females increasing continuous lighting with the entire visible spectrum to constant darkness. Spectral composition did not significantly influence sex ratio. Over the salinity range 26 ‰ to 44 ‰, light factors exerted a greater influence on the survival of *T. holothuriae* than salinity (Milou, 1993); temperature being the main factor influencing survival.

Researchers noticed that population density appeared to influence population growth. Fava & Crotti (1979) found that crowding of females during mating resulted in a reduced number of nauplii hatching from the first egg sac. Zhang & Uhlig (1993) determined that female fecundity is affected by both the density of females during their breeding phase and the density of nauplii during naupliar stages.

In addition to the influences of temperature, salinity, light and population density, diet exerts a major influence on *Tisbe* species growth and productivity. *Tisbe* species are known to demonstrate an opportunistic feeding behaviour utilising various food sources. Although *Tisbe* species will attempt to ingest anything remotely edible (Guidi, 1984) including microalgae, marsh grass, diatoms, algal biofilms, polychaete meat and mixed cereals, it has been suggested that food particle size should not exceed 250 µm (Guillet & Guérin, 1976 cited in Milou & Moraïtou-Apostolopoulou, 1991b).

Numerous researchers have successfully maintained populations of *Tisbe* species on a variety of foodstuffs including:

- bacteria (Vanden Berghe & Bergmans, 1981; Reiper, 1982; Guérin & Reiper-Kirchner, 1991),
- cyanobacteria (Lee et al., 1985),
- microalgae (Nassonge, 1970; Fava & Crotti, 1979; Lee et al., 1985; Webb & Marcotte, 1984; Marshall, 1993; Zhang & Uhlig, 1993; Norsker & Støttrup, 1994; Abu-Rezq et al., 1997),
- macroalgae (Guidi, 1984; Milou & Moraïtou-Apostolopoulou, 1991c; Marshall, 1993),
- vegetables (Kahan, 1979; Kahan et al., 1982; Chandler, 1986),

- processed plant products such as dried *Spirullina* and granular *Soya* (Milou & Moraïtou-Apostolopoulou, 1991c), and

- animal products such as artificial fish food, cat food, yeast, blood cells, liver, and dried mussel (*Mytilus*) and polychaete meat (Guérin & Kerambrun, 1982; Milou & Moraïtou-Apostolopoulou, 1991c; Marshall, 1993; Zhang & Uhlig, 1993).

The wide range of foodstuffs able to be used by species of *Tisbe* may be the result of the variety of feeding modes adopted by harpacticoids in general. Hicks & Coull (1983) reported the then unpublished work of Marcotte, who recognised two primary modes of feeding; surface browsing and filtering, each of which has subtypes. Surface feeding harpacticoids could be divided into four main categories: point-feeders, line-feeders, plane-sweepers, and solid-feeders. Representatives of *Tisbe* exhibit feeding modes within the solid-feeders category of which there are in turn three types: a) prey-crushers that grasp and devour prey with prehensile maxillae, b) sphere-cleaners that rotate spheres and organic floccules in their mouthparts cleaning food from the surface with well developed antennae, and c) rubble-sorters that pass organic debris over and through mouthparts where it is cleaned prior to passage posteriorly through the arched space between the swimming legs. True filter-feeding is not recognised among the majority of harpacticoids.

Tisbe appears to be capable of rejecting unpalatable food particles at any of three stages during the ingestion process (Marcotte in Hicks & Coull, 1983). The copepod can reject a food item after a) initial contact with the antennae; or b) after the particles had made contact with the maxillae and mandibles, or c) after mastication. Rejection is by violent reversed peristalsis of the foregut and appropriate manipulation by the mouthparts.

The feeding mechanisms adopted by various *Tisbe* species may encompass one or more modes categorised as solid-feeders explaining the varying degrees of success that have been experienced with the range of diets and the possibility of inter-species preferences observed by numerous researchers (Vanden Berghe & Bergmans, 1981; Rieper, 1982; Lee et al., 1985; Guérin & Rieper-Kirchner, 1991; Milou & Moraïtou-Apostolopoulou, 1991b; Marshall, 1993).

Vanden Berghe & Bergmans (1981) conducted a study to assess the feeding selectivity of three sympatric well-identified species of *Tisbe*: *T. holothuriae, T. battagliai* and *T. furcata*, using a radio labeling technique in combination with a monoclonal strain bacterium and an axenic microalga. *T. furcata* alone showed a preference for bacteria which was found to be reflected in its mandibular structure.

In contrast Abu-Rezq et al (1997) reared *Tisbe furcata* on the microalgae *Rhinomonas reticulata, Skeletonema costatum* and *Pavlova lutheri* observing poor

performance on the smallest microalga *P. lutheri* which they attributed to its small size and low energy content.

Further support for the more selective feeding habit of *Tisbe* species was provided by Lee et al. (1985). They observed *T. carolinensis* to perform better when provided exclusively with the diatom *Cylindrotheca* sp. than when fed the chlorophyte *Chlorella autotrophica* or the blue green algae *Agmenellum quadruplicatum* and *Anabaena* sp.. The higher *T. carolinensis* growth rates achieved on *Cylindrotheca* sp. were attributed to the larger particle size (4x15 µm compared with diameters between 1.5 to 6 µm) and the higher protein and lipid content of the pennate diatom (Lee et al., 1985).

In addition to growth rate, enzyme activity has also been shown to vary according to diet (Guérin & Kerambrun, 1982) as has biochemical composition.

The nitrogen and protein content of food sources affect development time, with no specific relationship identified between carbon and/or caloric content (Guidi, 1984). *Tisbe holothuriae* exhibits a characteristically high protein content (Milou et al., 1992) ideal for larviculture.

Similarly, the fatty acid PUFA profile of adult *Tisbe holothuriae* copepods was found to reflect the level of PUFA in their diet, but naupliar fatty acid PUFA composition was consistently high irrespective of the adult diet (Norsker & Støttrup, 1994). The ability of *Tisbe* to synthesise *n*-3 PUFA despite deficient dietary fatty acid content makes the species a potentially valuable live food organism for first feeding marine finfish larvae (Norsker & Støttrup, 1994).

Kahan et al. (1982) demonstrated that culture of the benthic *Tisbe* species could be restricted to the upper layer of the water column. Such techniques have been promoted for commercial use in the supply of naupliar feed to fish larvae present in the underlying tank. However the use of such floating basket cultures is not practical due to the subsequently reduced light levels and water quality in the larval rearing tanks.

Alternatively Støttrup & Norsker (1997) developed a production system for *Tisbe holothuriae* from which nauplii were harvested and successfully fed to turbot. Batch cultures relied on the use of flat trays with approximately 3 L media containing the microalgae *Rhodomonas* at approximately 1 million cells mL^{-1} producing 300 thousand nauplii per day. A complementary continuous system consisting of a closed recirculating copepod bioreactor with a 150 L volume filled with polypropylene balls was connected to a continuous microalgal cultivation system supplying *Rhodomonas*. Harvesting of nauplii was achieved by reversing the culture flow and using light to drive the copepodid stages against the water flow, or to hide in the culture substrate. The continuous method produced 250 thousand nauplii per day. The lower productivity of the continuous culture system

was attributed to early exhaustion of food supply. The use of *Tisbe* nauplii in turbot larviculture yielded the unexpected result that copepod nauplii increased the appetite of turbot larvae to twice that observed for rotifer-fed turbot (Støttrup & Norsker, 1997). Van der Meeren (1991) had previously observed turbot larvae to selectively ingest nauplii of harpacticoids when offered a mixture of rotifers and different stages of harpacticoid and calanoid copepods.

A natural extension of the Northern Hemisphere research is the use of southern temperate copepod species in the larval rearing of Southern Hemisphere flatfish. Individuals of the genus *Tisbe* are commonly seen in the greenback flounder broodstock and juvenile grow-out recirculating systems and they do not appear to have any adverse effect on the fish held in the respective systems.

The *Tisbe* species investigated here was found to be persistent in the recirculating systems at the University of Tasmania Key Centre for Aquaculture, Launceston, and the larval rearing systems at the Tasmanian Department of Primary Industries, Taroona Marine Laboratories, Hobart. The species is tolerant of a wide range of temperatures and salinities (Milou & Moraïtou-Apostolopoulou, 1991) and is often an inhabitant of tidal pools in association with the marine chlorophyte *Ulva* species (Barr, 1969 and personal observation).

Dr John Moverley, of the Crustacean Department of the Museum Victoria, Australia, identified the Tasmanian harpacticoid as a member of the genus *Tisbe*. No specific name was able to be assigned as a result of the taxonomic complexity of the genus and paucity of published work from the southern hemisphere (Prof JBJ Wells, Victoria University of Wellington, *pers. comm.*). Voucher specimens comprising 10 male and 10 ovigerous *Tisbe* and the details of their origin have been deposited with the Museum and Art Gallery of the Northern Territory, catalogue number NTM Cr012711.

Throughout the remainder of the thesis, unless indicated otherwise, the name *Tisbe* relates specifically to the Tasmanian species of the genus.

2.1.2 Objectives

The aim of the chapter is to document the culture characteristics of a temperate Australian *Tisbe* species and assess its suitability as an alternative life food for marine finfish larvae using greenback flounder as a test species. Three distinct aspects were investigated: (a) life cycle, (b) culture and (c) larval feeding trials.

The objectives of the work undertaken were to:

A. Document the number and sizes of each of the stages in the life cycle of *Tisbe*.

B. Describe the gross morphological features of each stage so that it is possible to differentiate developmental stages during culture.

C. Determine the mean generation time and duration of each of the life stages of *Tisbe.*

D. Determine the influence of light level on the distribution of *Tisbe* in culture.

E. Identify the influence of salinity, temperature, culture medium exchange rate, aeration and diet on the growth and development of *Tisbe.*

F. Assess the effects of salinity, temperature, culture medium exchange rate, aeration and diet on culture population density and demographic variables such as population structure, net rate of reproduction and sex ratio.

G. Identify whether greenback flounder larvae accept *Tisbe* nauplii and/or show a feeding preference for *Tisbe* in comparison to rotifers at first feeding.

H. Investigate the stage-related dietary preferences of flounder larvae.

2.2 Materials and methods

Research on *Tisbe* was undertaken in two locations. The majority of the work was completed at the University of Tasmania, Launceston campus, in temperate Tasmania. However as a consequence of my relocation during the course of study, the Life Cycle component of the chapter was completed in tropical Darwin, in the Northern Territory of Australia, with samples obtained from Tasmania.

Common methods

Throughout the investigation all water quality parameters were measured using the same equipment. Similarly, unless stated otherwise, the algae used were cultured under the conditions detailed below.

Water quality monitoring

Temperature, recorded in degrees Celsius (°C), was measured using a laboratory standard, calibrated mercury thermometer accurate to 0.1°C.

Salinity, recorded in parts per thousand (‰), was measured using an ATAGO S-10 hand-held refractometer accurate to 0.5 ‰ calibrated prior to use with temperature acclimated freshwater.

Dissolved oxygen levels (DO, $mgO_2\ L^{-1}$) were measured using a WTW Oxi320 oxygen meter accurate to within 0.2 $mgO_2\ L^{-1}$ calibrated daily according to the manufacturer's instructions.

pH levels, accurate to within 0.01 pH units, were measured using a Hanna Instruments pH meter (HI 8424) calibrated daily according to the manufacturers instructions.

Levels of nitrogenous compounds were determined using the corresponding HACH colourimetric test kits for salt water systems. The lowest level of nitrite (NO_2) detectable by the HACH test kit was 0.03 mg $NO_2\ L^{-1}$. The lowest level of unionised ammonia reportable, when appropriate conversion tables factoring the influences of pH and temperature on the percent ionisation of ammonia were used, was 0.10 mg $NH_3N\ L^{-1}$.

Algal culture

All species of microalgae were batch cultured in the University of Tasmania Aquaculture Centre algal laboratory at 26°C, 30 ‰, under a 14 L:10D photoperiod regime between 17,000 and 20,000 lux (330-590 $\mu mols^{-1}m^{-2}$) and fertilised with f_2 medium (Guillard & Ryther, 1962). Seawater for algal cultures was successively filtered through sand and then through 5 µm and 1 µm cartridge filters prior to being filtered to 0.1 µm in a sterilised drum filter.

Stock cultures were maintained in 200 mL flasks and scaled up through 500 mL flasks and 20 L polycarbonate carboys to 200 L plastic bags. Each 200 L culture was fed to rotifer and copepod cultures for a period of 2 to 3 weeks, during which time the bags were refilled with filtered seawater and refertilised as required.

Four species of single celled microalgae were used in subsequent trials with *Tisbe*:

a. *Tetraselmis suecica* (CSIRO collection no. CS187) referred to in remainder of the chapter simply as *Tetraselmis*. This prasinophyte exhibits cell lengths within the range of 12-15 µm.

b. *Isochrysis* species Tahitian clone (T-iso, CSIRO collection CS-177) referred to in remainder of the chapter simply as *Isochrysis*. This prymnesiophyte exhibits cell lengths within the range of 3-5 µm.

c. *Pavlova lutheri* (CSIRO collection no. CS182) referred to in the remainder of the chapter simply as *Pavlova*. This prymnesiophyte exhibits a cell length within the range of 4-6µm.

d. *Nitzschia palacea* (Northern Territory University collection no. NT7) referred to in the remainder of the chapter simply as *Nitzschia*. This bacillariophyte exhibits a cell length within the range of 17.5 x 12µm.

2.2.1 Life cycle and demographics

Knowledge of the life cycle of an organism and the characteristics of each life stage in the cycle is essential to the successful culture of the organism. *Tisbe* has not been used in the Australian aquaculture industry previously and is therefore unknown to the majority of aquaculturists. The objectives of the following investigations were to observe the life cycle of the Tasmanian *Tisbe* and identify key characteristics of importance to aquaculture technicians in the culture and use of *Tisbe* as a live food for finfish larviculture.

A mixed population of *Tisbe* was transferred by air from the University of Tasmania's Aquaculture Centre, Launceston, Tasmania to the Darwin Aquaculture Centre in the Northern Territory where a quarantined, lightly aerated 100 L culture was established. The culture was fed a mixed diet of *Tetraselmis* and *Isochrysis* to achieve a final cell density around 10^5 cells mL^{-1}, supplemented by a small amount of crumbled artificial barramundi food pellets. The *Tisbe* culture was maintained at 35 ‰ and 22 ±0.5 °C in a constant temperature room subjected to a 14 L:10D photoperiod and light intensity of 30 lux (0.6 µmol s^{-1} m^{-2}).

The stock culture was fed daily during a five day acclimation period after which time the majority of the ovigerous *Tisbe* present were known to be recently matured females (Marshall, unpublished). Using a Pasteur pipette ovigerous *Tisbe* were collected from the sides of the 100 L culture vessel and transferred to

individual 70 mL, cylindrical, clear plastic, individual culture units fitted with lids, each of which held 20 mL of fresh media with *Isochrysis* and *Tetraselmis* at 10^5 and 10^4 cells mL^{-1} respectively, at 35 ‰.

2.2.1.1 Life cycle

Eighteen individual culture units were subsequently maintained at a temperature of 22.7 °C (± 0.16°C; range: 21.4 to 24.7°C) by immersion in a water bath as depicted in Figure 2.2.1. The water baths consisted of a cylindrical, plastic 100 L Nally® bin filled to 90 L with water and heated using a 250 Watt Jagar aquarium heater. Aeration was supplied to each via a 4 mm air line fitted with a ceramic weight to prevent thermal stratification. Temperatures were recorded twice daily and any necessary adjustments made accordingly.

The eighteen ovigerous *Tisbe* cultures were monitored daily for the appearance and hatching of egg sacs by placing each individual culture unit directly on the stage of an Olympus SZ40 stereo dissecting microscope. Once all nauplii had hatched from each egg sac, the females were individually transferred to separate vessels containing fresh media, thus separating nauplii developing from successive egg sacs.

The hatched nauplii were monitored daily and allowed to develop for varying lengths of time to obtain representative specimens of each of the life stages for measurement at the appropriate magnification using an Olympus BH-2 compound microscope fitted with 4x, 10x, 20x and 40x objective lens in combination with 10x eyepieces. Illustrations of the twelve life stages were drawn free hand, highlighting the gross morphological changes readily visible under a dissecting microscope.

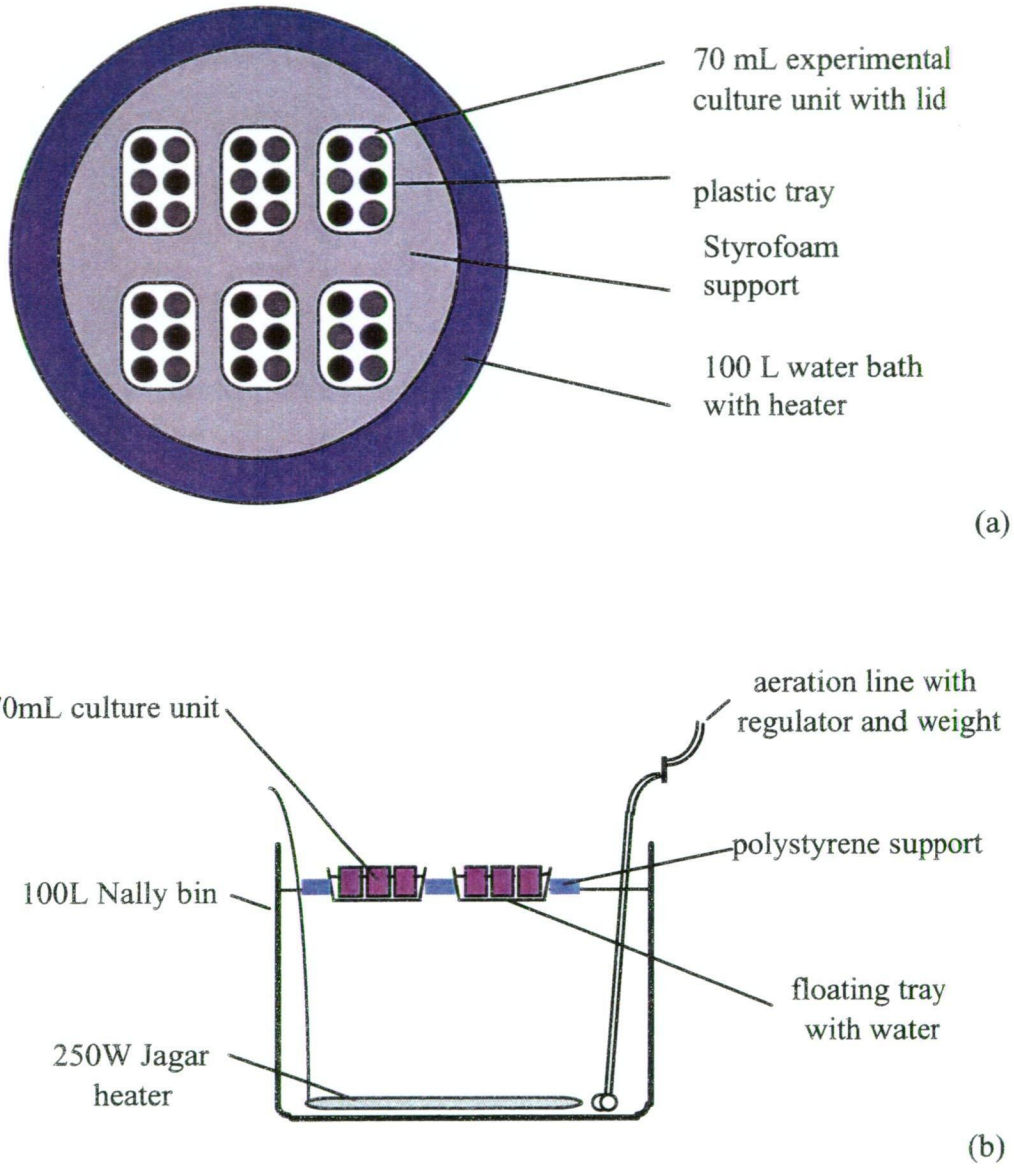


Figure 2.2.1: Diagram of the 100 L water bath.

(a) Aerial view of the system showing the individual culture units floated within a rectangular plastic tray in turn supported by a Styrofoam skirt floating in the 100 L Nally® bin.

(b) A profile of the same system depicting the position of the heaters and aeration with respect to the individual culture units.

2.2.1.2 Demographics and productivity

Fifteen ovigerous *Tisbe* were isolated from the quarantined population and maintained under identical temperature, salinity, photoperiod and diet conditions to those detailed above. The intended treatment temperature was 20°C, however the air conditioning unit in the constant temperature room used was able only to maintain a room temperature in the vicinity of 23°C. Previous experience with *Tisbe* had indicated a tolerance of temperatures up to 27 °C (Marshall, unpublished).

All fifteen *Tisbe* females were monitored daily for the extrusion and hatching of egg sacs. Upon hatching of each egg sac, the females were individually transferred by Pasteur pipette to fresh culture media in separate 20 mL vessels. The hatched nauplii were retained in the initial culture vessel and assigned a label comprising the numeric female identifier and a serial alphabetic egg sac identifier. For example, the first clutch of nauplii hatched from female 5 was labelled 5a, the second egg sac to hatch 5b and so on.

Developing progeny from each individual egg sac were observed daily in the same manner as the females, and the stage of development corresponding to that stage reached by 50% of the individuals during the preceding 24-hour period recorded. F_1 females were removed from the progeny cultures upon the appearance of their first egg sac to prevent the hatching of a second generation of nauplii in the 20 mL culture. The F_1 females were also monitored for frequency of egg sac extrusion, progeny development and labeled in a similar fashion to F_0 females and progeny. The total number of nauplii developing through to maturity, the sex ratio, and male longevity were also documented.

Initial female fecundity was assessed from a separate sample of 20 ovigerous *Tisbe* obtained from the same stock culture as the inoculum F_0 females. Length measurements and the number of eggs carried were determined under an Olympus BH-2 compound microscope.

The filial generations were monitored for 2 months. F_0 females were those females initially isolated from the imported population. F_1 females were those females of the first filial generation obtained in culture and F_2 the second filial generation developing from egg sacs of F_1 females.

The demographic variable of mean generation time (T) was obtained from direct observation of populations developing in individual culture units. Indicative natural rate of intrinsic increase (r) and net rate of reproduction (R_o) were calculated according to Milou & Moratiou-Apostoloupoulou (1991a,b) as outlined in the General Introduction (Section 1.4.3).

Statistical analyses

Statistical analyses were employed to ascertain the value of body size of each life stage as a diagnostic characteristic. The dimensional data collected for each stage was subject to Shapiro-Wilk's test for normality and Bartlett's test for homogeneity of variance prior to the completion of analyses of variance (ANOVA). Scheffe's multiple means comparison test was used to identify significantly different means revealed by ANOVA.

Where data were unable to be transformed to meet the underlying assumptions of ANOVA, these were analysed using the non-parametric Kruskal-Wallis *k*-sample test, Mann-Whitney *U*-test and Tukey's multiple means comparison test where appropriate. All transformed data were converted back into original units of measurement for presentation in supporting figures and tables.

All statistical analyses completed for inclusion in this thesis, were without exception, conducted in Statview® and JMP® software designed for use with Macintosh computers.

2.2.2 Culture of *Tisbe*

The experiments conducted in this section were designed to quantify a number of the culture characteristics exhibited by *Tisbe*. Previous experience by myself with this copepod (Marshall, 1993) had revealed it to be tolerant of a high degree of handling, to tolerate a wide range of environmental conditions and to exhibit a strong negative phototactic response.

The following experiments were conducted to quantify the phototactic response and the influence of water exchange, aeration, salinity, temperature and diet on the population dynamics of *Tisbe*.

Small volume cultures without substrate were used to render these experiments easier to maintain, and ensure that population density estimates were not confounded by uneven extraction of experimental animals from the substrate, or uneven distribution of food.

Stock culture maintenance

Tisbe populations comprising stock culture A were maintained at 20 °C (range 18 to 21 °C) on a mixed diet of *Tetraselmis* and *Isochrysis* and mussel purée (the meat of *Mytilus planulatus* macerated in 1 µm filtered seawater and kept in the refrigerator). The original inoculum for the population were collected from the recirculating systems for flounder broodstock maintained at the University of Tasmania's Aquaculture Centre, Launceston, Tasmania.

The *Tisbe* inoculum for stock culture B were obtained from the same recirculating systems revisited two years later. *Tisbe* stock cultures were maintained in a

moderately aerated 100 L culture at 35 ‰ and 16 °C on a mixed algal diet of *Tetraselmis, Isochrysis* and *Nitzschia.* The *Tisbe* stock was not maintained at the ideal culture temperature of 20 °C as it had been observed that at 16 °C copepod productivity was greater than that of contaminant rotifers yielding a cleaner *Tisbe* inoculum.

The original natural inoculum was derived from *Tisbe* collected with the seawater at Weymouth off the north coast of Tasmania. The seawater is trucked 50 km inland from the coast to the University Aquaculture Centre where all marine systems are closed, or semi-closed recirculating systems. The water is stored in completely closed 20 m^3 fibreglass tanks prior to being pumped to various areas of the Aquaculture Centre.

2.2.2.1 Phototaxis

The later stage copepodids of *Tisbe,* which were readily seen with the naked eye against the side of stock culture vessels, were observed to be more dense near surface areas of lower light intensity. The following trial was designed to quantifying the phototactic response of all life stages of *Tisbe* against the hypothesis that light level had no influence on copepod spatial distribution.

A mixture of *Tisbe* adults, copepodids and nauplii was obtained by screening a volume of the stock culture over a 36µm-mesh screen. *Tisbe* individuals retained on the screen were rinsed with 1 µm filtered seawater prior to resuspension in fresh filtered seawater at 30 ‰. *Tisbe* life stages were added to the two aquaria at one of two densities: 300 individuals L^{-1} or 600 individuals L^{-1}.

Two rectangular plastic 1 L aquaria (29 cm long, 9 cm wide and 7 cm deep) were filled with 1 µm filtered seawater at 30 ‰ to a depth of 6 cm (total volume 0.9 L). A triple layer of black shade cloth was placed over one half of each aquaria (covering 14.5 cm) with the light source directly overhead establishing a fully light area (A), a transition zone (B) and a shaded area (C) (Figure 2.2.2). Light levels in each of the three areas decreased from 100 lux in area A, through 30 lux at the transition zone B to 3 lux in C.

Food was excluded from the system to eliminate aggregation of copepods due to feeding, thus removing a potentially confounding influence. The effect of surface attachment was not addressed directly, however the aquaria were recently cleaned in hot water and air-dried thus removing any bacterial film which might have encouraged surface association.

The aquaria were left undisturbed for 7 hours after which time two 20 mL samples were drawn from the middle of the water column in each of the three areas in both aquaria. Combined, the samples comprised 12% of the total culture volume.

A χ^2 -test was applied to the counts data to test the null hypothesis that light had no influence on copepod distribution. ANOVA was also conducted to assess the existence of any light, density or life stage interactions. Normality of data and homogeneity of variance were tested using, respectively, Barlett's and Shapiro-Wilks analyses. The data collected were found not to require transformation.

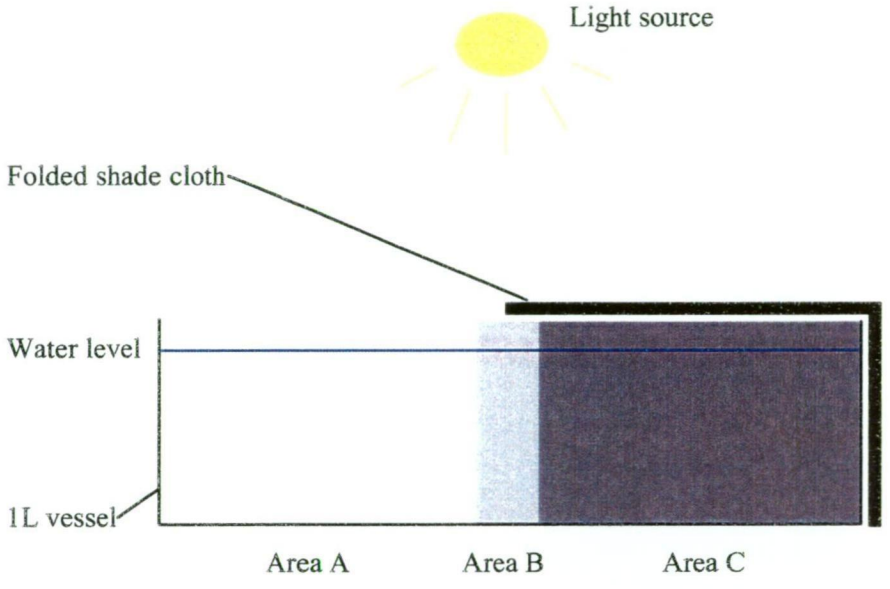


Figure 2.2.2: Diagram of the experimental tank used to quantify the phototactic response of *Tisbe* illustrating the light zonation along the length of the 1 L aquaria. The fully lighted Area A experienced a mean light level of 100 lux, the transition zone Area B experienced 30 lux, and the shaded Area C received 3 lux.

2.2.2.2 The effects of salinity and aeration

The effect of aeration on 500 mL *Tisbe* culture populations was tested in triplicate at three salinities: 25, 35 and 45 ‰. The three salinities were chosen as a result of preliminary trials assessing salinity tolerances of ovigerous *Tisbe* (Appendix A2), the range tested also corresponding to the most likely range of salinities at which live foods would be required for marine finfish larvae.

Four litres of culture media was made up at each of the three treatment salinities of 25, 35 and 45 ‰ using the appropriate combination of 1 µm filtered seawater of 35 ‰, deionised water and sodium chloride. *Isochrysis*, *Pavlova* and *Tetraselmis* at had a final density of approximately 10^5 cells mL^{-1}.

Six culture units were filled with 500 mL of media for each of the three treatment salinities. Three culture units at each of the treatment salinities were supplied with air via a regulated 4 mm plastic line terminating in a glass Pasteur pipette. Airflow was adjusted to deliver two bubbles every second. The combinations resulted in three replicates per treatment: 25 ‰ without aeration, 25 ‰ with aeration, 35 ‰ without aeration, 35 ‰ with aeration, 45 ‰ without aeration, and 45 ‰ with aeration.

Three hundred and sixty ovigerous *Tisbe* were transferred from a stock culture (maintained as described above) by screening a portion of the culture over 150 µm-mesh and rinsing with 1 µm filtered seawater. Each female was isolated and rinsed by pipetting her through a series of fresh seawater droplets to reduce contamination. Such treatment has previously been determined not to affect the long-term vitality of the copepods. Twenty ovigerous *Tisbe* were transferred using Pasteur pipettes to each of the eighteen culture units which were subsequently randomly distributed on a shelf in a temperature control room maintained at approximately 20 °C.

Culture units were checked daily to ensure aeration was maintained and the ambient air temperature recorded. Preliminary observations had shown the temperature of the media in 500 mL cultures to be the same as ambient air temperatures eliminating the need to record the temperature in each individual culture.

The trial was run for a period of 21 days. Every third day prior to feeding, a 10 mL sample was removed from each culture unit using a standardised protocol. Each culture unit was swirled 6 times in the same direction to homogenise the contents. A 10 mL sample was then removed from one-third of the way across the culture vessel, in the centre of the water column using a 10 mL syringe. Samples were labelled and preserved in a 1:1:8 mixture of formalin, glycerol and culture media. Post-sampling a further 50 mL of culture media was removed using a syringe covered by 36 µm-mesh to prevent removal of copepod stages with the culture

media. Each culture was subsequently fed 0.3 mL of mussel purée and 70 mL of algal mix of the appropriate salinity.

At the end of the trial, the number of ovigerous females, female (CVI), male (CVI), immature copepodid (CI-CV), late naupliar (NIV-NVI) and early naupliar (NI-NIII) *Tisbe* stages present in each preserved sample were counted using an Olympus SZ40 stereo dissecting microscope.

2.2.2.3 The effect of water exchange

Regular water exchange assists the removal of metabolites known to regulate copepod density (Fava & Crotti, 1979) and also assist in the mixing of culture medium, exerting a beneficial influence on culture population health and productivity (Vijverberg, 1989). The aim of the trial was to assess the level of benefit afforded to *Tisbe* culture population density by four rates of water exchange: 1) no water exchange, 2) once weekly water exchange, 3) twice weekly water exchange, and 4) daily water exchange.

500 mL of 1 µm filtered seawater at 35 ‰, 0.05 mL of mussel purée and a light mix of *Pavlova*, *Isochrysis* and *Tetraselmis* was added to culture units to achieve a final cell density of approximately $1x10^5$ cells mL^{-1}. Light aeration was provided to each culture unit on the basis of results from the preceding trial.

Three hundred and sixty ovigerous *Tisbe* were transferred from stock culture A by screening a portion of the culture over a 150 µm-mesh screen and rinsing with 1 µm filtered seawater. Twenty females were then individually transferred using Pasteur pipettes to four replicate culture units for each treatment which were randomly distributed on a shelf in a room with ambient temperature maintained between 18.5 and 20 °C.

Water exchange involved siphoning 200 mL (40 %) of the culture medium from each culture unit through a 36 µm-mesh screen to prevent removal of any *Tisbe* life stages. The 200 mL was replaced with 120 mL of acclimated 1 µm filtered seawater adjusted to 35 ‰, 80 mL of algae mixture and 0.2 mL of mussel puree. Every third day all cultures were fed 0.4 mL of mussel purée. Every seventh day all cultures received 50 mL of a mixture of *Tetraselmis*, *Isochrysis* and *Pavlova*.

The trial was terminated after 15 days at which time the contents of each culture unit was collected on a 36 µm-mesh screen and preserved in a 1:1:8 mixture of formalin, glycerol and seawater with the number of individuals in each life stage group determined as detailed in Section 2.2.2.2.

2.2.2.4 The effects of temperature and salinity

The objective of this trial was to measure any change in *Tisbe* numbers in association with salinity and temperature combinations in 200 mL cultures fed a

mixed diet of algae and artificial crumbled fish food when maintained at combinations covering the range from 15 to 40 ‰ and 15 to 30 °C.

Culture units filled with 200 mL of seawater were placed in Contherm® heating/cooling water baths. The thermostats of the water baths were adjusted to maintain culture unit water temperatures at 10 °C, 15 °C, 20 °C, 25 °C and 30 °C (±0.1 °C).

A total of 350 ovigerous *Tisbe* were pipetted individually from the sides of the 100 L Nally® bins of *Tisbe* B stock cultures and rinsed in 1 µm filtered seawater. Each 200 mL culture unit was inoculated with five ovigerous *Tisbe.*

At the commencement of the trial (D0), 4 L of each of the 5 culture media at six salinities 15, 20, 25, 30, 35 and 40 ‰ were made up with *Isochrysis* and *Tetraselmis* at densities of $1x10^5$ and $6x10^4$ cells mL^{-1} respectively. Algal culture densities for *Tetraselmis* and *Isochrysis* in 180 L bags were estimated from the average of four to six haematocytometer counts. The culture media was acclimatised to the ambient temperature of the stock culture (16°C) prior to distribution between the corresponding 150 mL cylindrical plastic culture units. Five grains of the Lansy® artificial diet NRG4, weighing approximately 1.2 mg, each, were added to each of the ninety-six culture units.

The 120 culture units were randomly allocated between the five water baths maintained at each of the five temperatures: 10, 15, 20, 25 and 30 °C, such that each salinity was represented in quadruplicate at each temperature.

Temperature, salinity, pH and dissolved oxygen levels of the cultures were monitored daily with light levels recorded at the commencement and termination of the trial. Salinity fluctuations due to evaporation were kept to a minimum through the addition of distilled water where necessary. Food levels were adjusted on Mondays and Thursdays to ensure consistency between treatment temperatures and salinities.

F_0 females were not removed from the culture vessels as the experiment aimed to assess the overall effect of temperature and salinity on overall productivity. The trial was terminated on day nine (D9) allowing sufficient time for nauplii hatching from inoculum ovigerous *Tisbe* to develop through to maturity. Environmental parameters were recorded prior to all cultures being screened separately over a 44 µm mesh. The copepod stages retained on the mesh of the screen were preserved in a 1:1:8 mixture of formalin, glycerol and culture medium with the number of individuals in each life stage group determined as detailed in Section 2.2.2.2.

2.2.2.5 The effect of diet

Tisbe species are well known as omnivores (Hicks & Coull, 1983; Milou, 1993). The aim of the following trial was to assess the usefulness of diets readily available

in aquaculture facilities and with which aquaculture technicians would be familiar. The high proportion of algal-based diets assessed reflects the prevalence of these in aquaculture facilities and the associated benefits imparted by algae in rearing systems in terms of their probiotic effects, ability to improve or maintain the condition of culture medium and nutritional value (Kellam & Walker, 1989; Støttrup et al., 1995).

Preliminary trials assessing the influence of larval artificial fish feed and the benthic diatom *Nitzschia* on *Tisbe* productivity (see Appendix A4) recognised the potential suitability of these food sources in the culture of the harpacticoid. The following trial compares the relative productivity of the two 'new' food sources with that achieved when *Tisbe* cultures were fed the traditional microalgal diets used with rotifers and *Artemia*.

The influence of diet on *Tisbe* culture productivity was assessed in terms of the number of individuals developing in six replicate culture units over nine days when presented one of the seven diets detailed in Table 2.2.1.

Ovigerous *Tisbe* were removed from the *Tisbe* B stock culture by siphoning the sides of the culture using a length of 4 mm plastic tubing. Three hundred and sixty *Tisbe* females were rinsed in 0.2 µm filtered seawater prior to inoculation of experimental culture units.

A partial flow-through system was used to minimise the effects on the system of deteriorating algal quality and metabolite build up over time. Each culture system consisted of cylindrical 500 mL vessel gravity fed by a 4 mm weighted delivery line and flow regulator from a 1 L reservoir at a rate of 300 mL hr^{-1}. Excess media exited the culture vessel via an overflow covered by a 44 µm screen to exclude copepods (Figure 2.2.3).

Two litres of culture media were made up for each of the algal diets at a salinity of 35 ‰ and allowed to equilibrate to 20 °C. A 500 mL volume of each of the six diets were distributed to the corresponding six replicate culture units. The six unfed replicates were filled with 500 mL of filtered seawater. Each of the thirty-six culture units were inoculated with ten ovigerous *Tisbe* on day zero (D0) prior to random distribution between two blocks in a room with temperature control maintained at 20 °C.

Table 2.2.1: Composition of the seven experimental diets presented to *Tisbe* for a period of nine days to assess their influence on culture productivity. Iso – corresponds to *Isochrysis*, Nitz – corresponds to *Nitzschia* and Tet – corresponds to *Tetraselmis*.

Diet	*Isochrysis* (cells mL^{-1})	*Tetraselmis* (cells mL^{-1})	*Nitzschia* (cells mL^{-1})	Lansy® (grains)
1. unfed	-	-	-	-
2. Tet & Iso	$1.0x10^5$	$6.0x10^4$		-
3. Nitz	-	-	$1.0x10^5$	-
4. Lansy® crumble	-	-	-	25
5. Tet, Iso & Nitz	$2.0x10^4$	$3.3x10^4$	$2.0x10^4$	-
6. all components	$1.5x10^4$	$2.5x10^4$	$1.5x10^4$	5

Temperature, salinity, pH and dissolved oxygen levels of the cultures were monitored daily prior to exchange of media. The contents of each 1 L reservoir were replenished on a daily basis with the corresponding fresh media to facilitate at 60% exchange over 3 hours. Prevailing light levels were recorded at the commencement (D0) and termination of the trial (D9).

The trial was terminated after nine days (on D9). Environmental parameters were recorded for all cultures prior to the addition of formalin and glycerol. The persevered *Tisbe* were collected over a 44 µm mesh screen and retained in 20 mL of a 1:1:8 mixture of formalin, glycerol and culture medium with the number of individuals in each life stage group determined as detailed in Section 2.2.2.2.

Statistical analyses

All environmental parameters and *Tisbe* population data collected were subjected to Shapiro-Wilk's test for normality and Bartlett's test for homogeneity of variance. Non-normal data were transformed using an appropriate method prior to the completion of analyses of variance. Treatment effects in normal data exhibiting homogenous variance were determined by analysis of variance (ANOVA) and Scheffe's multiple means comparison tests.

Data unable to be transformed to meet the assumptions of ANOVA were analysed either using Kruskal-Wallis *k*-sample test or Mann-Whitney *U*-test in conjunction with Tukey's multiple means comparison test. All transformed data were converted back into original units of measurement for presentation in supporting figures and tables. Culture population density data are reported as the equivalent number of *Tisbe* L^{-1} to enable comparison between trials conducted in culture units of different volumes.

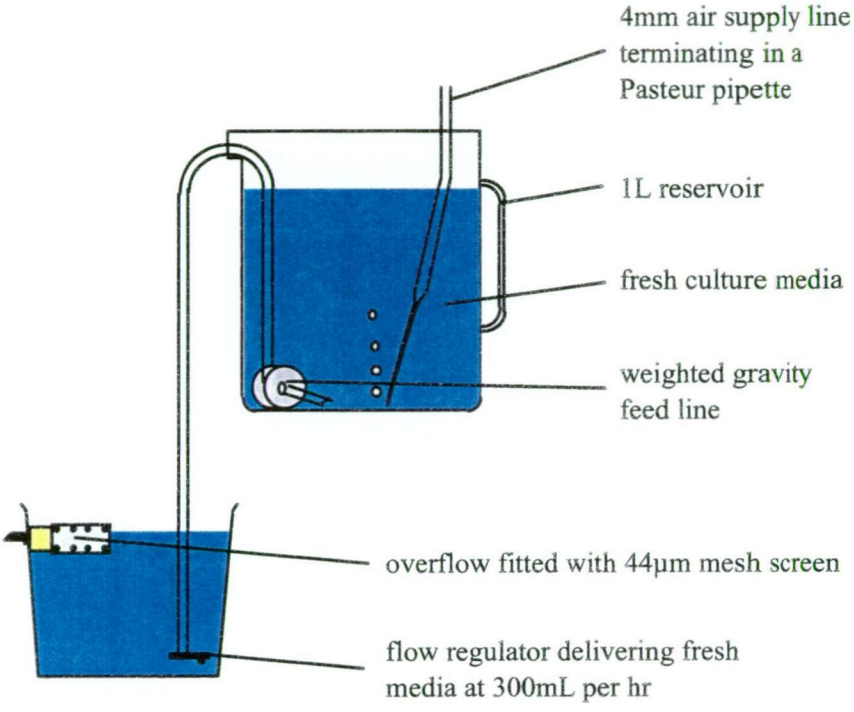


Figure 2.2.3: Diagram of the culture system used to asses the influence of diet on *Tisbe* culture density consisting of a 500 mL culture unit (CU) fitted with a 44 µm mesh screened overflow and a 1 L reservoir.

2.2.3 Larval fish feeding trials with *Tisbe*

Short-term trials were conducted to assess the effect of handling, preservation and exposure to live food organisms on the gut contents of flounder larvae. Subsequent trials investigated live food preferences and feeding behaviour displayed by the flounder of different ages.

The intended end point of the series of trials was the completion of a medium-term (35 day) performance trial assessing the survival, growth and development of flounder larvae reared on traditional rotifer and *Artemia* diets compared to larvae reared on a diet of copepods.

The feeding trials conducted with *Tisbe* and flounder follow a natural progression through the development of techniques for the handling of both the finfish larvae and harpacticoid life stages to optimise larval feeding incidence during the trials investigating flounder feeding preference at various stages during their development.

Common methods

Detailed below are the techniques and protocols used to culture and handle the live foods and flounder larvae used in feeding trials. The details of the various experimental larval rearing systems and water quality monitoring equipment are also described.

Water quality assessment

All measurements were taken using the equipment detailed in the Common methods of Section 2.2.

Temperature and salinity were measured at the time each trial was inoculated, and after the completion of each trial. Random checks of pH, dissolved oxygen, ammonia and nitrite levels were also completed to ensure no contamination of water, algae or live food cultures proved a confounding factor in any of the trials.

The low stocking densities of copepods and fish larvae used in conjunction with the short-term nature of the trials meant that rigorous testing for nitrogenous compounds was not necessary.

Flounder larvae production

Flounder eggs were obtained either from naturally ripe females or females artificially induced using 'Ovaprim' (Hart, 1994). Eggs and sperm were hand stripped from ripe females and males. Eggs were fertilised using seawater-activated sperm. The fertilised eggs were incubated at 12 °C in 160 L black, hemispherical fibreglass tanks under a 18 L:6D photoperiod and hatched five days

later designated day zero, (D0). Water temperatures were subsequently increased gradually from 12 °C to 15 °C as recommended by Hart (1994).

Flounder larvae commenced exogenous feeding on the fourth and fifth day post-hatch (dph) at which time rotifers were introduced at a density of 10 mL^{-1}. Larvae were fed rotifers at an increasing density (10 to 20 rotifers mL^{-1} day^{-1}) until the larvae reached a mean size of 4 mm (usually 8 to 10 dph) at which time *Artemia* were introduced at 1 mL^{-1}.

Usually rotifer densities were reduced to 10 mL^{-1} once the flounder larvae had metamorphosed at approximately 15 dph, and the density of *Artemia* increased to a density of 3 mL^{-1}.

The juvenile flounder reared for the purposes of these trials were not weaned onto artificial diets as is usual at 23 dph (Hart, 1994), so that live food experimentation could be extended. Rotifers continued to be supplied at a density of 5 mL^{-1} and the density of *Artemia* (enriched instar II metanauplii) fed to the growing larvae gradually increased. Flounder of 33 dph were fed *Artemia* at 15 mL^{-1}.

In an attempt to minimise any confounding environmental stresses, all feeding trials were conducted in the same insulated room with temperature control and an 18 L:6D photoperiod in which the flounder were hatched and reared. Water temperatures prevailing during trials ranged from 12 to 15 °C according to the developmental stage of the flounder.

Rotifer production

Large strain rotifers (*Brachionus plicatilis*) were maintained in well-aerated 500 L tanks on a mixed diet of Baker's yeast (*Saccharomyces cerevisiae*) and the algae *Isochrysis* and *Nannochloropsis oculata* (CSIRO collection number CS179) at 25 ‰ and 25 °C. Water exchanges (20 % of the total volume) were conducted on alternate days using *N. oculata* culture as the replacement for extracted water.

Twenty-four hours prior to being used in larval feeding trials, the required number of rotifers plus 20% were concentrated and washed over a submerged 63 µm mesh screen. Rotifers were rinsed in freshwater for 1 minute to reduce ciliates prior to 24 hours enrichment using a mixture of *Pavlova* and *Tetraselmis* at a final algal cell density of 10^5 cells mL^{-1}.

On the morning of the flounder larvae feeding trials, the enriched rotifers were collected in water over a wet 63 µm mesh screen, rinsed and resuspended in clean seawater at 35 ‰, counted and adjusted to a density of 130 to 150 rotifers mL^{-1}. The prepared rotifers were left for 1 hour in an aerated vessel to temperature acclimate in the larval rearing room prior to feeding at a final density of 10 mL^{-1} to the flounder larvae.

Copepod production

Tisbe cultures were maintained in cylindrical, food-grade plastic 100 L Nally® bins provided with moderate aeration (approximately 300 mL minute^{-1}) on a mixed diet of algae (*Isochrysis*, *Pavlova*, *Tetraselmis*) and mussel purée (the meat of two mussels *Mytilus planulatus* pureed with 20 mL of distilled water). Water exchanges were conducted weekly on the cultures maintained at 32 ‰ (±3 ‰) and 18 °C (±1 °C).

Four days prior to the commencement of larval feeding trials, ovigerous females were isolated by screening a portion of the stock culture over a 212 µm mesh screen. The late stage copepodids retained were rinsed in fresh seawater of 35 ‰ prior to being added to 50 L of fresh culture media at 35 ‰ with algae and mussel puree. The salinity of 35 ‰ had previously been identified as favouring the production of *Tisbe* nauplii (Section 2.3.2.2).

On the morning of the feeding trials, the contents of the four day old copepod culture was screened over nested 37 µm, 125 µm and 212 µm mesh screens. The size fraction of copepods used in the larval feeding trials was determined by the age of the flounder larvae used in the feeding trial (Table 2.2.2) corresponding to increasing mouth size.

Table 2.2.2: *Tisbe* life stages fed to flounder of various ages (dph - days post-hatch).

Size Fraction (µm mesh)	*Tisbe* Life Stages	Age of flounder larvae
37 - 125	NI - NIV	5 - 8 dph
125 - 212	NV - CIV	9 - 15 dph
>212	CV - CVI	> 16 dph

The appropriate size fraction of *Tisbe* was collected on the morning of the feeding trials. The *Tisbe* were subsequently cleaned and resuspended in clean seawater at 35 ‰, counted and adjusted to a density of 130 to 150 individuals mL^{-1}. The copepods were left in the larval rearing room for 1 hour in an aerated vessel to temperature acclimate prior to being presented to the flounder larvae at a final density of 10 mL^{-1}.

Artemia production

Artemia cysts (Prime®) were decapsulated using a 2-3% active hypochlorite solution, rinsed thoroughly, and hatched in 20 L conical plastic 'socks' with vigorous aeration 48 hours prior to feeding. *Artemia* nauplii thus hatched were

subsequently enriched for 12 hours with Frippak®, according to the directions on the packaging.

On the morning of the feeding trials, the enriched *Artemia* metanauplii (instar II) were rinsed in freshwater and resuspended in clean seawater at 35 ‰ at a density of 130 to 150 mL^{-1}. The prepared *Artemia* were left in the larval rearing room in an aerated vessel for 1 hour to temperature acclimate prior to feeding at a final density of 10 mL^{-1} to the flounder.

The mini-aquaria (500 mL)

The mini-aquaria used were 500 mL cylindrical food grade plastic containers spray-painted black on the outside to simulate conditions in the 160 L black hemispherical larval rearing tanks. The mini-aquaria were located in the larval rearing room under the same photoperiod, light intensity and temperature conditions as the stock larval rearing system.

Mini-aquaria were filled with aerated, temperature acclimated 1 µm filtered seawater (35 ‰). No water exchange was conducted during trials in the mini-aquaria due to their short duration (<36 hours) combined with the relatively low larval stocking densities and large surface area to volume ratios of the mini-aquaria.

The small scale system (3 L aquaria)

The small scale aquaria consisted of up to twenty black, plastic round bottomed containers spray-painted black on the outside to simulate light and prey contrast conditions experienced by larvae in the 160 L black hemispherical larval rearing tanks.

Each aquarium was filled with 2.5 L of temperature acclimated, aerated 1 µm filtered seawater at 35 ‰ seawater obtained from the larval rearing system reservoir the evening prior to the feeding trials.

The setup was located in the larval rearing room and was also static over the 36 hour experimental period.

The 'cover-slip-squash' technique

The number of live food organisms from the gut contents of individual flounder was determined for larvae euthanased by an overdose of anaesthetic. A cover slip was applied at the head of the larva and laid down over the body towards the tail, assisting the evacuation of gut contents which were subsequently counted under a stereo-dissecting microscope.

Gut contents of flounder larvae were recorded as the number of rotifers, *Tisbe* life stages or *Artemia* nauplii identifiable once evacuated from the larval digestive tract.

This technique was used effectively with larvae of 1 to 14 dph corresponding to larval stages 1a, 1b, 1c, 1d and 2b as described by Crawford (1984).

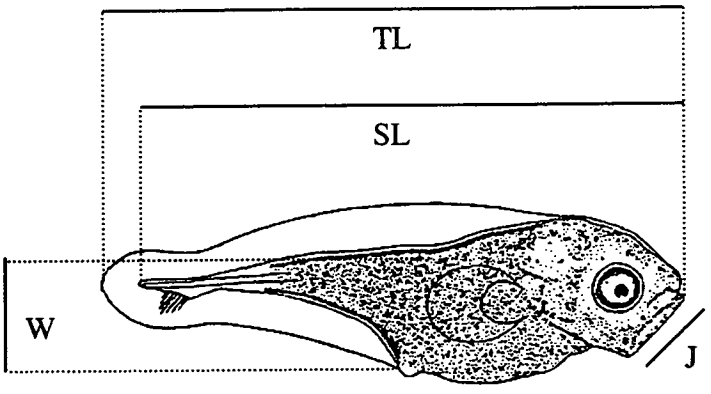
Further development of the larva is associated with increased complexity of the digestive tract and pigmentation over the gut area. Consequently the gut contents of older larvae and juveniles, corresponding to larval stages 3a, 3b, 4a and 4b, were counted by first dissecting out the gut, achieved by cutting the notochord and pulling the stomach away from the pigmented area. The cover slip was then applied directly to the dissected stomach.

Larval length measurements

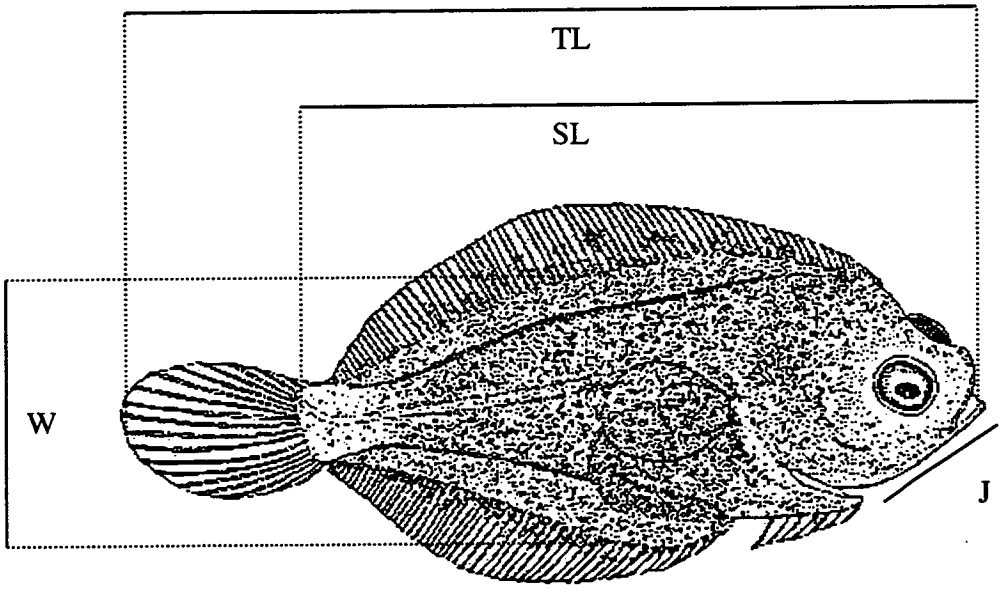
Four measurements illustrated in Figure 2.2.4, were recorded for larvae used to describe the flounder used in the feeding trials: standard length (SL), total length (TL), body width (W) and lower jaw length (J).

Standard length was recorded as the distance from the base of the caudal fin to the end of the upper lip, with total length measured from the end of the caudal fin to the end of the upper lip of larvae. Lower jaw length was measured from the tip of the lower lip to the angular return on the jaw.

Two general morphologies were identified to reflect the change in body form associated with the development of a flat fish species. In larvae less than 15 dph, body width was recorded as the distance across the larval body from a point immediately behind the vent to the base of the dorsal fin. In larvae of 16 dph and older, body width was recorded from the widest point behind the vent to the edge of the dorsal fin.



(a)



(b)

Figure 2.2.4: Explanation of the lines of measurement recorded for each of the four parameters taken for flounder larvae of various ages: total length (TL), standard length (SL), body width (W) and lower jaw length (J).
a) depicts the points of measurement in pre-metamorphosis larvae 4 to 15 days post-hatch (from Crawford, 1984),
b) the corresponding points in flounder larvae 16 dph and older.

Chesson's Index of larval food selectivity

The selectivity index (α) is used to compare the relative feeding preferences of greenback flounder larvae for each live food item. Larvae that did not feed were excluded from the analysis because they had not made a live food selection. The α value was calculated using the equation by Chesson (1978):

$$\hat{\alpha} = \frac{\frac{r_i}{n_i}}{\sum_{j=1}^{m} \frac{r_j}{n_j}} \qquad i = 1,\ldots,m$$

where r_i is the number of prey items of live food type *i* in the larval diet (m= total number of live food types) and n_i is the number of items of prey type *i* in the environment. Both r_i and n_i can be expressed as the percentage of prey type *i* in the diet and environment, respectively, without affecting the value of α.

Significant differences in preference (α) on a mixture of two live food types (species *a* and species *b*) were analysed using a *t*-test comparing α on species *a* to the natural selection level of α = 0.5 (50% probability of consuming 1 of 2 live food types if no prey preference is operating) on each day the trial was run, using the equation:

$$t = \frac{(\alpha_i - 0.5)}{\sqrt{\frac{s^2}{k}}}$$

where α_i is the sample mean and s^2 is the sample variance of k estimators (n) of α_i. A significant positive or negative preference for species *a* means that there is a significant preference in the opposite direction for the alternative live food type (species *b*) in the mixed diet. Since one *t*-test per mixed diet treatment was calculated for each trial day, alpha levels of $p<0.001$ were considered significant.

Analyses of significance for α for mixed diets containing 3 live food types were calculated using the same equations as above, with the modification that the neutral selection level was adjusted to 0.33 to reflect the one in three chance of a larva selecting a particular live food type in the absence of prey preference. As more than one *t*-test is required per mixed diet treatment each trial day, it was necessary to adjust the significance level using the Dunn-Sidak adjustment to maintain the Type 1 error at 0.001. The appropriate alpha level for a mixed diet comprising three live food types is therefore $p<0.003$.

2.2.3.1 Initial feeding trials

The following three trials were conducted to assess the effects of handling on the early developmental stages of flounder, the success of transferring embryonic flounder to experimental aquaria for later use in feeding trials, and to determine the potential of *Tisbe* as a live food for flounder larvae. The larvae used in the trials were introduced to the experimental systems on the day prior to the application of the experimental treatments consequently the larvae used to inoculate the experimental systems were, in fact, one day younger than the larvae exposed to the treatments.

Trial 1.1 – The effect of flounder age on the ingestion of Tisbe *life stages*

The aims of the trials were to:

- assess the resilience of flounder eggs and larvae of 7 dph and 21 dph to handling during transfer from larval rearing tanks to experimental aquaria,
- assess the suitability of the 3 L aquaria as experimental tanks, and
- determine larval acceptance of Tisbe life stages as a live food for flounder larvae.

Thirty flounder eggs at the tail-bud stage were individually transferred to each of fifteen static 3 L aquaria using a wide bore plastic pipette (Ø 3 mm). Hart (1994) identified the tail-bud stage as the earliest embryonic developmental stage resilient to handling. The eggs were left to hatch in the aquaria, with subsequent larvae left undisturbed. At 3 dph a noticeable oil film had covered the air-water interface. The film was removed by the careful application of an absorbent paper to the water surface.

When larvae were 5 dph and exogenous feeding was imminent, live food preferences were assessed by presenting the larvae with one of five live food combinations: 1) rotifers, 2) *Tisbe* nauplii (stages NI-NIV), 3) *Artemia* nauplii and 4) a mixture of the three live foods, presented at a final density of 10 mL^{-1}, and 5) an unfed control. Three replicate aquaria were randomly assigned to each treatment.

The 5 dph larvae were left to feed for 2 hours prior to being anaesthetised using a 200 ppm benzocaine solution and transferred to 5% buffered formal saline with length measurements and gut contents recorded at a later date.

Flounder larvae hatching from the same batch of eggs as the 5 dph larvae used in the preceding trial were maintained in dedicated 160 L hemispherical larval rearing tanks on a diet of rotifers since commencement of feeding. At 7 dph twenty of these larvae were transferred to each of the fifteen 3 L aquaria using wide bore plastic pipettes the evening prior to the trial, and left to purge overnight. At 10:00

hours the following morning, the preference of the 8 dph larvae was assessed using the same four live food combinations and unfed control as for the 5 dph larvae. After 2 hours exposure to the treatment diets, the 7 dph larvae were processed in the same manner as the 5 dph flounder.

Two weeks later, twenty flounder of 21 dph reared on a mixed diet of rotifers and *Artemia* in the dedicated 160 L larval rearing system were transferred to each of the fifteen 3 L aquaria in the same manner as the 7 dph larvae, and left to purge overnight. At 10:00 hours the following morning, the 22 dph larvae were exposed to single live food diets and an unfed control. *Tisbe* copepodids were used instead of nauplii reflecting the larger mouth size of the 22 dph flounder. After 2 hours exposure to the treatment diets, the 22 dph larvae were processed in the same manner as the 5 dph and 7 dph flounder.

Trial 1.2 – Larval feeding incidence as influenced by flounder age and live food type

Trials were conducted with 8 dph and 26 dph flounder to: a) assess larval feeding success in the mini-aquaria, and b) observe flounder larvae of 8 dph and 26 dph actually feeding on *Tisbe*,

Fifteen 7 dph flounder larvae were individually pipetted from the 160 L larval rearing tank into each of the eight mini-aquaria using a wide bore plastic pipette. The 120 flounder larvae were subsequently left undisturbed for 17 hours overnight in the hatchery to purge.

On the morning of the trial, a low concentration of *Tetraselmis* (10^4 cells mL^{-1}) was added to each of the eight aquaria to simulate green-water larval rearing conditions. Flounder in duplicate aquaria were then presented with one of four treatment diets, the final density of the live foods adjusted to 10 individuals mL^{-1}: 1) an unfed control, 2) rotifers, 3) copepod nauplii, and 4) *Artemia* (instar II).

The larvae were left to feed undisturbed after which time all fifteen were individually pipetted onto a glass slide and their gut contents counted using the cover-slip-squash technique. The exposure time varied within each treatment from 4.5 to 6 hours as a result of an unexpected increase in processing time as a result of sampled larvae being processed immediately without preservation as it had been demonstrated preservation reduced the number of prey items identified within the larval gut.

The data collected were averaged to obtain the mean number of live food organisms (LFO) per larva, the number of larvae feeding, the number of mortalities and the number of larvae 'missing' (that is larvae known to have been added but not recovered at the end of the trial).

The same process was repeated with founder of 25 dph from the same batch which had been reared in the same larval tank and fed rotifers and *Artemia.*

Subsequent trials indicate that the size of the flounder in the sample did not differ between diet treatments at each age. Gut content data from larvae were log transformed prior to the completion of ANOVA with all figures converted back to true units prior to inclusion in figures and tables.

2.2.3.2 Experimental trials with first feeding larvae

The following trials were conducted with first feeding flounder larvae from three separate batches of eggs to assess the significance of stress-induced prey evacuation from the larval gut, the optimal live food exposure time, and prey preference when flounder larvae are presented a mixed diet at the onset of exogenous feeding. All the experiments were conducted in 3 L aquaria located in the same temperature control room maintained at 12 °C (±0.5 °C) with a 18 L:6D photoperiod as the stock larval rearing tank.

Trial 2.1 - Effects of feeding duration and larval preservation on gut content retention

Twenty-five 4 dph larvae were individually transferred from the 160 L larval rearing tank using a wide bore (∅ 3 mm) plastic pipette to each of twenty 3 L aquaria prepared with 2.5 L of 0.1 µm filtered acclimated seawater at 35 ‰ and left to acclimate overnight. The following morning, rotifers were presented at a density of 10 mL^{-1} and the 5 dph larvae left to feed for 1, 2, 3, 4, 29 and 30 hours.

After the designated feeding time had expired, larvae were individually pipetted from the corresponding replicates and euthanased by anaesthetisation in a 200 ppm benzocaine solution. Ten of the anaesthetised larvae were immediately preserved in 5% buffered formal saline. An additional ten larvae were placed onto microscope slides without being preserved. Total length, body width and jaw length measurements were recorded for each larva prior to subjecting the larva to gut content assessment using the cover-slip-squash technique. The same information was recorded for the ten preserved larvae from each replicate 48 hours later.

Gut content data collected exhibited a normal distribution with equal variances after being transformed using a square root function. No significant interaction was identified between feeding duration and preservation treatments.

Trial 2.2 - Live food preference of flounder at first feeding

The previous trial did not provide a clear indication as to the optimal feeding duration for the determination of gut contents of first feeding larvae for the interim time frame between 4 hours and 29 hours feeding duration. The results from a preliminary trial conducted using a mixture of live food species identified 6 hours

as the most practical exposure time; allowing sufficient time to achieve a high percentage of feeding success and greater number of live food items per gut, and still leave time for sampled larvae to be processed. This trial was designed to investigate the existence of live food species preference by flounder at the first feeding.

Twenty 4 dph flounder larvae were individually transferred to each of twelve 3 L aquaria as previously described. The following morning three replicate aquaria were inoculated with one of four treatments: 1) an unfed control, 2) *Tisbe* nauplii, corresponding to the 37 to 125 µm size fraction, 3) rotifers and 4) a 1:1 mixture of *Tisbe* nauplii and rotifers. All live foods were presented at a final density of 10 mL^{-1}.

The first feeding larvae were left undisturbed for six hours before processing as results from the preliminary trials indicated that six hours exposure would maximise the number of prey items likely to be found in the gut contents. Total length, body width and jaw length measurements taken prior to gut content assessment using the cover-slip-squash technique. Gut content data (both raw and transformed) failed tests of normality and homogeneity of variance and were subsequently analysed using the Kruskal-Wallis *k*-sample test.

2.2.3.3 Trials with 8 day post-hatch larvae

Older larvae have greater predatory and digestive capabilities (Blaxter 1988; Osse, 1990). The following trials aim to assess the effect of larval development on the gut evacuation reflex response, optimal exposure time and the expression of larval live food preference.

Trial 3.1 - The effect of live food type and preservation on larval gut content

Twenty 7 dph flounder were transferred to each of twelve 3 L aquaria and left overnight as described previously. On the morning of the feeding trial *Tisbe* nauplii corresponding to the 37 to 125 µm size fraction, and rotifers were prepared. Three replicates of the four diets were inoculated: 1) an unfed control, 2) *Tisbe* nauplii, 3) rotifers and 4) a 1:1 mixture of nauplii and rotifers. All prey organisms were presented at a final density of 10 mL^{-1}.

The 8 dph flounder were left undisturbed for a period of 6 hours. The twenty larvae in each replicate aquaria were randomly distributed between the fresh and preserved treatments. Ten larvae were processed fresh with total length, body width and jaw length measurements taken prior to gut content assessment using the cover-slip-squash technique. The other ten larvae were preserved in 5% buffered formalin saline with length measurements and gut content data collected within 24 hours of preservation.

The gut contents data were analysed using non-parametric statistics as the data were unable to be transformed to meet the assumptions of normality or homogeneity of variance.

Trial 3.2 – The effect of feeding duration on larval gut content

The previous trial revealed preservation of larvae did not affect the number of live food items recorded from the gut contents of larvae sampled. The convenience afforded by delaying the processing of samples to the day following the completion of the trial provided the opportunity to incorporate either greater replication or additional treatments into experimental designs. This trial was conducted to identify the most appropriate food exposure time to

Ten 7 dph larvae were transferred to a 3 L system overnight to purge their gut contents. On the following morning, rotifers were presented at a density of 10 mL^{-1} and the larvae left undisturbed under standard conditions in the larval rearing room for either 1, 2, 3, 4, 5 or 6 hours prior to removal and euthenasing, with total length, jaw length and gut contents were determined as per previous trial.

2.2.3.4 Trials with 15 and 25 day post-hatch larvae

These trials were designed to assess the influence of physiological development and increased predatory experience with exposure to additional prey items, as the flounder used had been exposed to a third live food since 12 dph.

Twelve 500 mL mini-aquaria containing 150 mL 0.1 µm filtered seawater at 35 ‰ were prepared and placed in the constant temperature room at 15 °C (± 0.4°C). Fifteen 14 dph flounder were individually transferred into each of the twelve mini-aquaria and left to purge over night (18 hours).

At 10:00 hours on the following morning, five treatment diets were randomly assigned to three replicate mini-aquaria: 1) unfed control, 2) rotifers, 3) *Artemia* (instar II), 4) *Tisbe* and 5) a mixture of three live food species in a 1:1:1 ratio. All live food prey items were presented at a final density of 10 mL^{-1}. The *Tisbe* size fraction used comprised those individuals retained on the 125 and 212 µm mesh screens corresponding to late stage copepodids including ovigerous females.

The larvae were left to feed undisturbed for 2 hours after which time the replicates were systematically processed: larvae were individually caught and euthanased with total length, body width and jaw length measurements taken prior to gut content assessment using the cover-slip-squash technique.

The same protocol was repeated with flounder of 25 dph sourced from the same stock tank. The gut contents data recorded were analysed using non-parametric analyses as the data were unable to be transformed to meet the assumptions of normality or homogeneity of variance.

2.2.3.5 Live food preference as influenced by larval flounder age

The preceding trials were undertaken on an opportunistic basis. However, the following trials were designed to obtain time series information from larvae sourced from the same batch of eggs, and reared in the same larval tank from the commencement of exogenous feeding (5 dph) through to post-metamorphosis (33 dph). The preferences of a subsample of larvae removed from the rearing tank were assessed on a weekly basis. The flounder larvae in the stock tank were exposed to the feeding regime described in the Common methods Section 2.2.3.

On a weekly basis, fifteen 3 L aquaria were filled with 2.5 L of 0.1 µm filtered seawater at 35 ‰ the afternoon prior to the commencement of the feeding trial. Thirty flounder were pipetted individually from the 160 L black hemispherical tanks in to each of fifteen aquaria using a wide bore plastic pipette (Ø 3 mm).

The larvae were left overnight to purge and settle. At 10:00 hours on the morning of the trial, the live foods were prepared and live food combinations introduced to three replicate aquaria corresponding to the five treatments: 1) rotifers, 2) *Artemia*, 3) *Tisbe*, 4) a 1:1:1 mixture of rotifers, *Artemia* and *Tisbe*, and 5) unfed control. Prey items were presented at a final density of 10 mL^{-1}. The life stage of *Tisbe* presented to the larvae differed with the age of the flounder as outlined in the Common Methods Section of 2.2.3.

Larvae were left to feed for 3 hours then removed individually from the aquaria and euthanased prior to transfer to a 5% solution of buffered formal saline. Total length, body width and lower jaw length measurements, and gut contents were assessed at a later date.

The feeding trial was completed with larvae of 5 dph, 12 dph, 19 dph, 26 dph and 33 dph.

Statistical analyses

All larval length, gut content counts and environmental parameter data collected were assessed for compliance with the assumptions underlying ANOVA.

Data sets not meeting the assumptions of normality and homogeneity of variances as determined by the application of the Shapiro-Wilk's test for normality and Bartlett's test for homogeneity of variances, were transformed using either square root, log or arcsine transformations as appropriate, to meet the assumptions and analyses conducted using the transformed datasets. The results of the analyses were converted back to original units for presentation in figures and tables.

Data unable to be transformed to meet the assumptions underlying ANOVA were analysed using Kruskal-Wallis and Mann-Whitney non-parametric statistical analyses to quantify treatment differences and Tukey's multiple means comparison test to identify significantly differing means.

2.3 Results

2.3.1 Life cycle and demographics

2.3.1.1 Life cycle

Twelve distinct stages possessing significantly different dimensional and morphological characteristics were identified in the life cycle of *Tisbe* (see Tables 2.3.1 and 2.3.2, and Figure 2.3.1).

Tisbe eggs are roughly spherical and approximately 60 µm in diameter. *Tisbe* nauplii only hatched successfully from the single egg sacs that remained attached to the genital somite of the female urosome. Nauplii did not hatch from egg sacs discarded as a result of shock from a sudden change of temperature, salinity, rough handling or physical removal. *Tisbe* females, from which egg sacs had been removed, (either as a result of shock or by gentle tugging using fine forceps), continued to extrude successive viable egg sacs under favourable conditions.

Table 2.3.1 documents the mean sizes of the six nauplius stages of *Tisbe*, and Table 2.3.2 presents the dimensions of the five sexually immature copepodid stages, and gender-specific details for the sexually mature adult copepodid stage CVI. Analyses of variance of length data revealed all life stages to be highly significantly different ($p<0.0001$), thus identifying length as a useful characteristic to differentiate between the life stages of *Tisbe* obtained from populations maintained under similar conditions.

The progressive differentiation of the telson of nauplii through the six naupliar stages are illustrated in Figure 2.3.1. All nauplii are dorso-ventrally flattened, and unpigmented except for the small, red, anterior naupliar eye. The general shape is circular as evidenced by length to width ratios (Table 2.3.1).

Further increase in segmentation following metamorphosis associated with development through the six copepodid stages are represented in Figure 2.3.2. The anatomical difference between the adult male and female *Tisbe* urosome structures and gender-related size disparity are highlighted. The copepodids of *Tisbe* possess an elongated, dorso-ventrally flattened body comprising a cephalosome, metasome (4 somites), urosome (5 somites) and caudal furca. All *Tisbe* copepodid stages are unpigmented, except for the occasional appearance of orange lipid droplets in the cephalothorax, and the red, anterior eyespot.

Sexual dimorphism is obvious by copepodid stage CVI, with females 21% larger than males. Anatomical differences are also evident in the antennule morphology and urosomal segmentation of adult male and female *Tisbe* (Figure 2.3.3 a and b). The antennules on the male are more stout than those on the female. Both antennules of the male are geniculate and play an important role in the grasping of the female during spermatophore transfer. The importance of these morphological

differences in the act of copulation depicted in Figure 2.3.3.c. Sexual dimorphism at copepodid stage CV, although evident, is not as obvious under a dissecting microscope as that observed in copepodid stage CVI.

Table 2.3.1: Dimensions (mean ±SE) of the six nauplius stages (NI-NVI) of *Tisbe*, where L corresponds to length as measured from the anterior to the posterior of the body, and W to cephalosome width at the widest point. L:W is the ratio of the total length to cephalosome width. SE indicates standard error, and n the sample size.

Life Stage	L ±SE (µm)	W ±SE (µm)	n	L:W
N I	80 ±0.4	67 ±1	12	1.2
N II	98 ±0.5	78 ±1	11	1.3
N III	136 ±1	108 ±2	10	1.3
N IV	156 ±1	117 ±3	10	1.3
N V	175 ±2	129 ±3	9	1.4
N VI	197 ±3	142 ±6	10	1.4

Table 2.3.2: The dimensions (mean ±SE) of the six copepodid stages (CI-CVI) of *Tisbe,* where L corresponds to total body length measured from the rostrum to the last urosomal segment, and W corresponds to cephalosome width at the widest point. L:W is the ratio of total length to cephalosome width. SE indicates standard error, and n the sample size.

Life Stage	L ±SE (µm)	W ±SE (µm)	n	L:W
C I	258 ±8	133 ±6	9	1.9
C II	326 ±11	129 ±3	10	2.5
C III	375 ±2	153 ±4	9	2.5
C IV	430 ±7	170 ±4	10	2.5
C V m	540 ±10	182 ±4	10	3.0
C V f	653 ±10	215 ±3	11	3.0
C VI M	648 ±12	253 ±4	13	2.6
C VI F	825 ±12	355 ±6	37	2.3

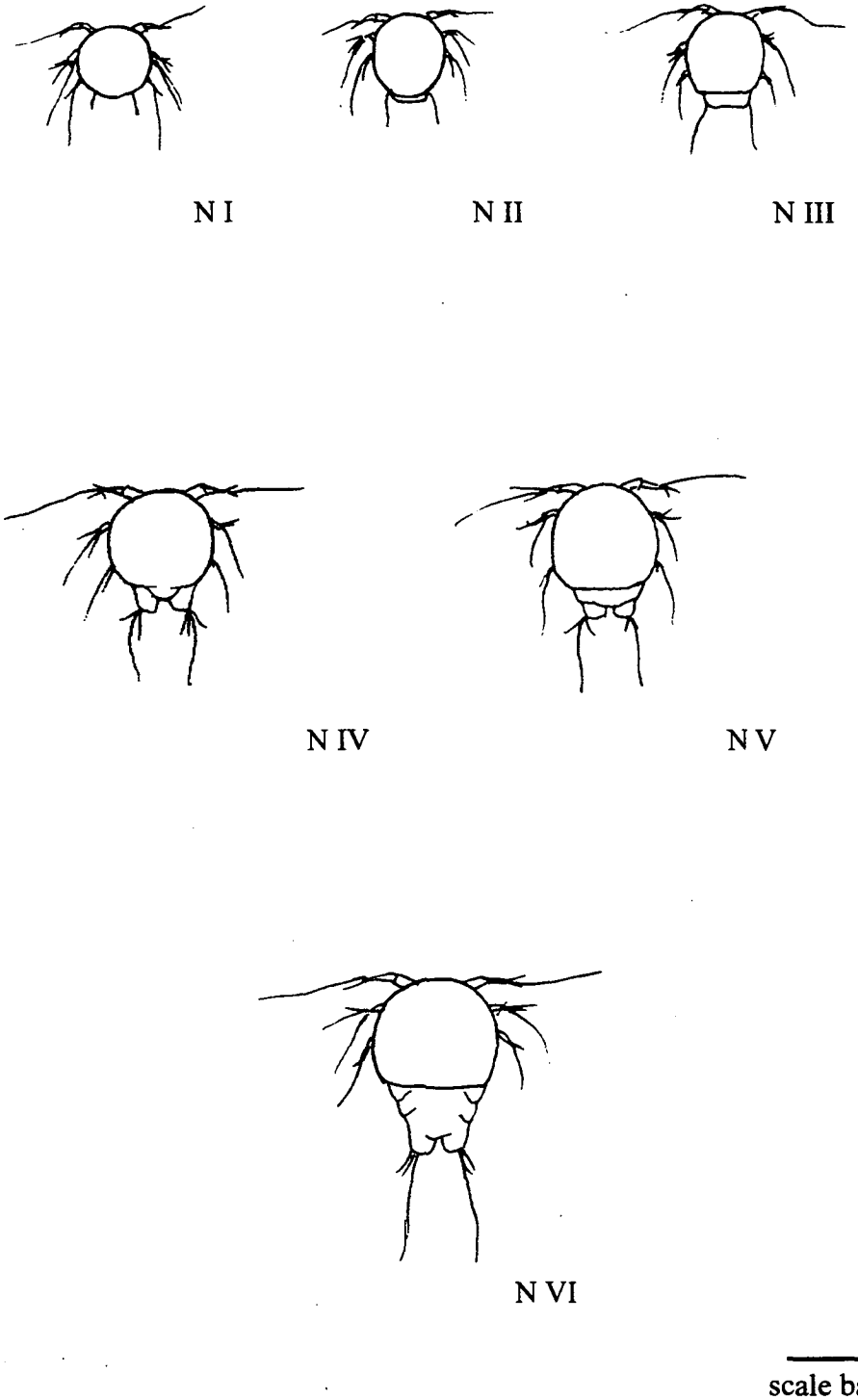


Figure 2.3.1: Illustrations of the six nauplius stages (NI through NVI) of the Tasmanian *Tisbe* isolate. Scale bar is 50 µm.

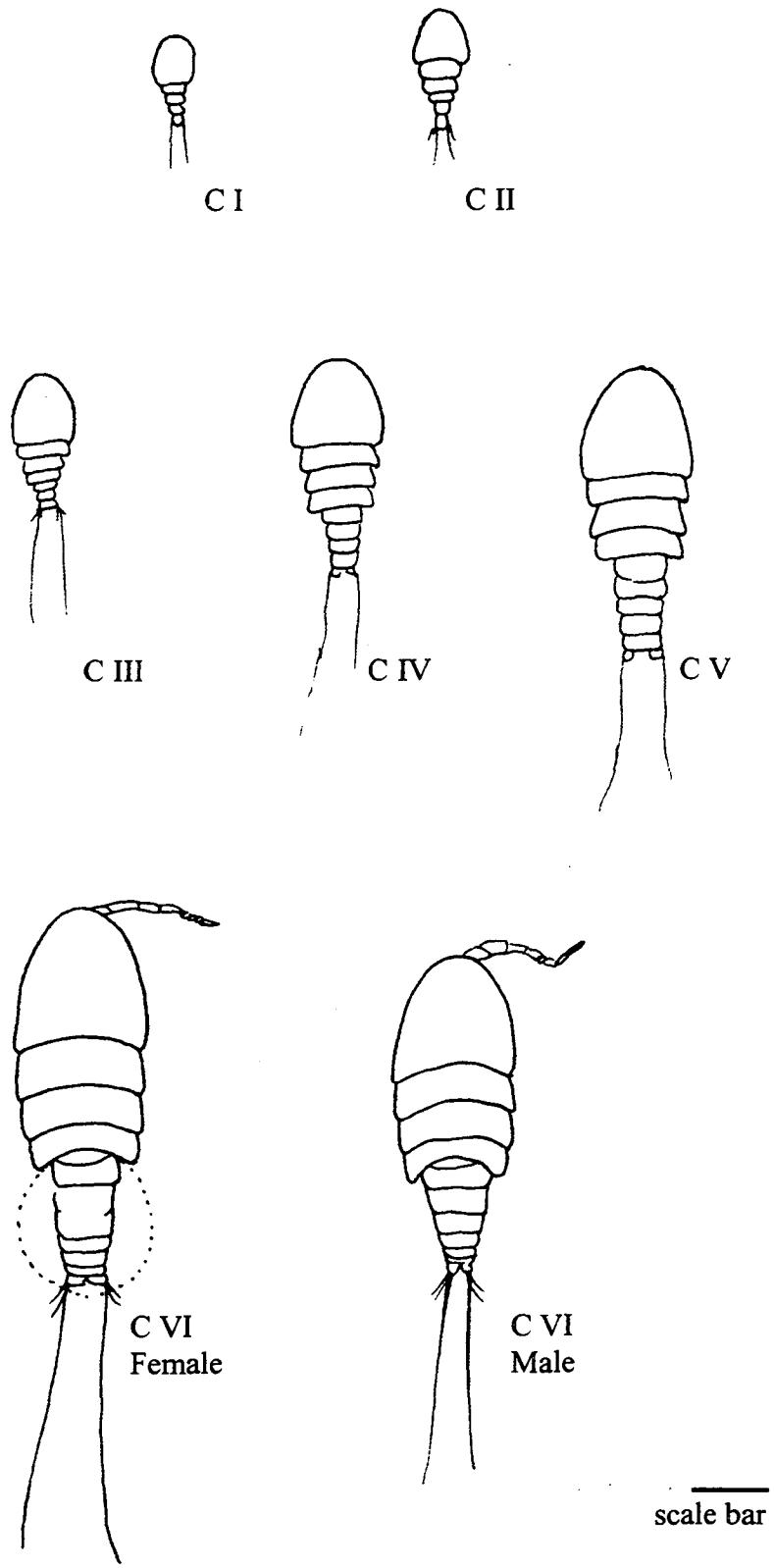


Figure 2.3.2: Illustrations of the six copepodid stages (CI through CVI) of the Tasmanian *Tisbe* isolate. Scale bars is 100µm.

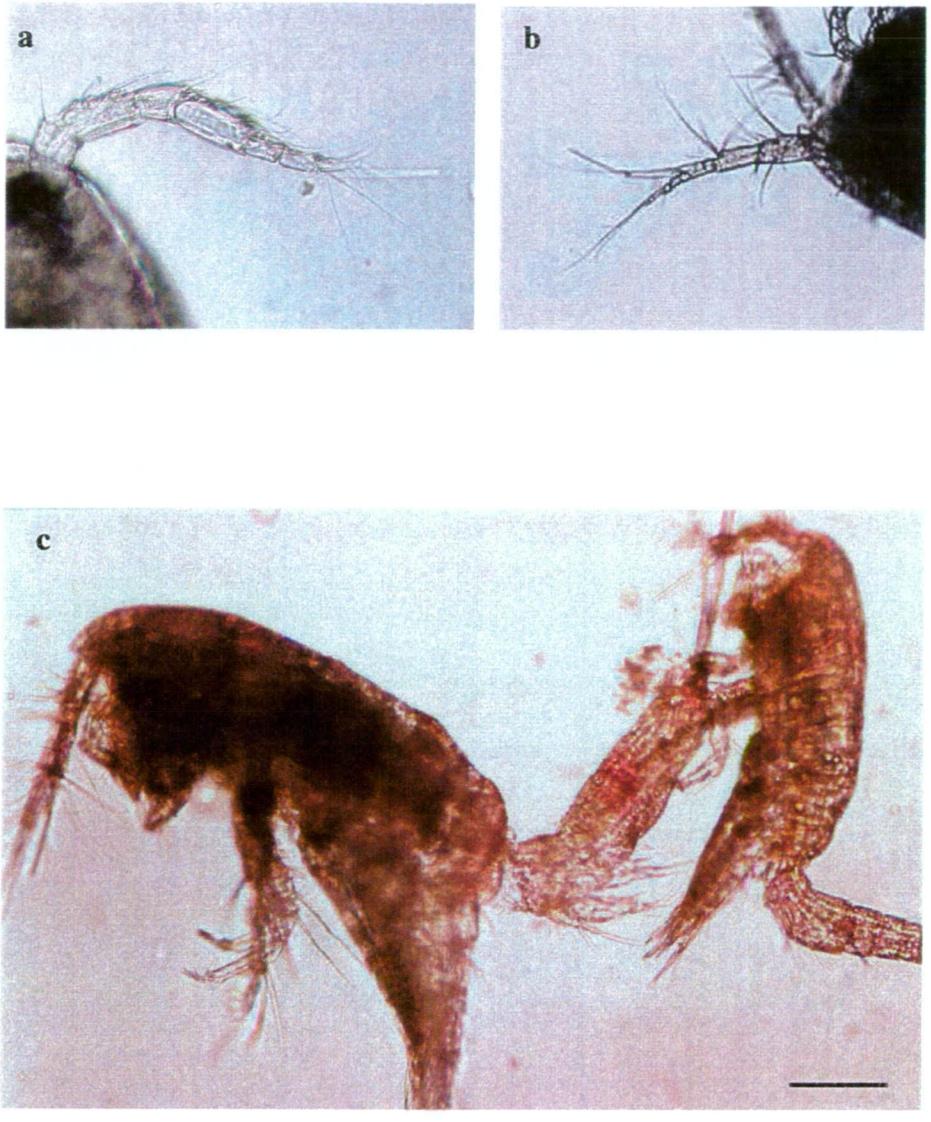


Figure 2.3.3: Photomicrographs illustrating the sexual dimorphism evident between the antennules of sexually mature copepodid CVI of the Tasmanian isolate of *Tisbe*.

a) male antennules each ~ 300µm in total length (x100)

b) female antennule each ~ 340µm in length (x100)

c) the importance of male antennule structure and the orientation of both male and female *Tisbe* during copulation. Scale bar is 100 µm.

2.3.1.2 Demographics and productivity

The mean generation time of *Tisbe* maintained at 22.7 ±0.16 °C (range 21.2 - 23.0 °C) in small volume cultures displayed a significant increase from 7.0 ±0.2 days to 7.9 ±0.2 days ($p<0.01$) as the cultures aged. This increase in generation time was also associated with a significant ($p<0.001$) decrease in the net rate of reproduction (R_o calculated as the ratio of third generation females to second generation females) from 9.6 ±1.8 in the original stock to 4.3 ±1.4 in subsequent generations.

The indicative intrinsic rate of natural increase, calculated as the ratio of natural log of *Ro* to the observed *T* also experienced a significant ($p<0.05$) decrease from 0.29 ±0.05 to 0.12 ±0.05 with prolonged culture. Additional information collected is recorded in Tables 2.3.3 and 2.3.4.

The average duration for both naupliar and copepodid stages was in the order of 12 hours. The time required for female *Tisbe* to receive spermatophores from mature males, and then extrude egg sacs is approximately 1.5 to 2.0 days. Egg sacs were extruded every two to three days thereafter, with nauplii observed to hatch between 1.5 and 2.0 days later.

Table 2.3.3: Demographic information for *Tisbe* cultures maintained at 21.7°C on a diet of *Isochrysis* and *Tetraselmis*. Figures are expressed as mean ± standard error, or percentage.

Parameter	F_0	F_1	F_2
Female longevity (days)	11.5±0.9	16.3±1.7	-
Egg sacs per female	4.2±2.2	5.1±0.5	-
% egg sacs hatch	27.9±4.1	76.0±5.0	-
% hatched egg sacs maturing	39.0±5.8	12.3±3.3	-
% aborting as nauplii	0.6±6.0	74.1±5.5	-
Net Rate Of Reproduction (R_0)	9.7±2.2	4.3±1.4	-
Mean Generation Time (*T*)	-	7.0±1.6	7.9±0.2
Sex Ratio	-	0.6±0.1	1.3±0.4
Male longevity (days)	-	22.9±1.4	19.2±1.1
% F_0 producing F_1 females	66.7%	-	-
% F_1 producing F_2 females	-	40.0%	-

Table 2.3.4: Observations of F_1 *Tisbe* females detailing differences between development of progeny from the first and second egg sacs extruded. Figures are expressed as mean ± standard error.

Parameter	1st egg sac	2nd egg sac
Sex ratio (Female:Male)	0.5 ±0.09	0.6±0.02
Nauplii duration (NI-VI)	2.6±0.1	3.0±0.0
Copepodid duration (CI-VI)	4.0±0.1	4.4±0.2
Mean generation time (T)	6.6±0.2	7.4±0.2

2.3.2 Culture of *Tisbe*

2.3.2.1 Phototaxis

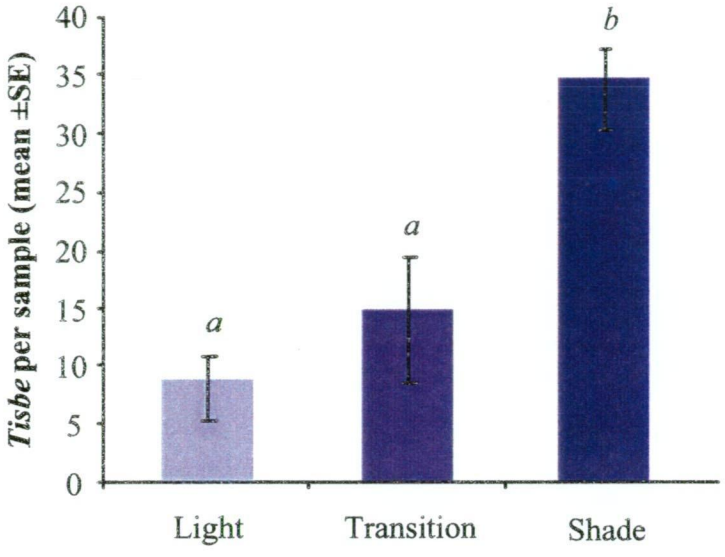
A strong negative phototactic response was exhibited by all life stages supporting the rejection of the null hypothesis that light has no effect on copepod distribution (Table 2.3.5). The data therefore confirm that all stages of *Tisbe* exhibit a negative phototactic response (Figure 2.3.4a).

ANOVA of the duplicate samples revealed that the number of nauplii, copepodids and adults were all significantly different between the zones in full light and under shade (Figure 2.3.4b).

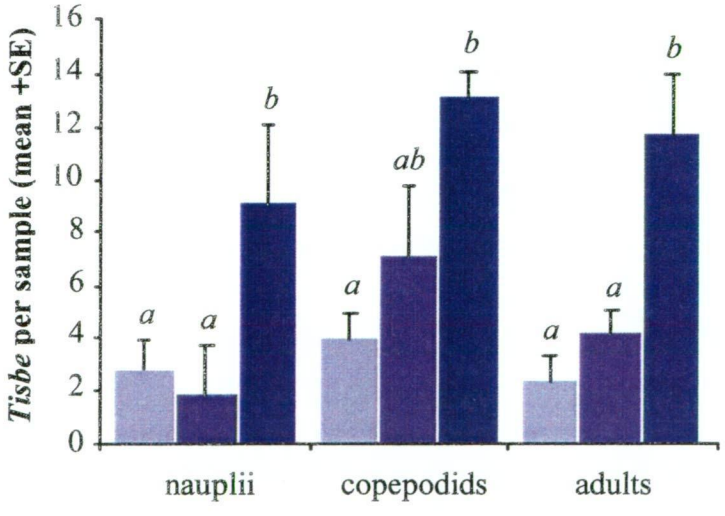
ANOVA did not identify a significant interaction between light level and culture density treatments with regard to the expression of negative phototaxis ($p>0.05$). However, the more dense the copepod culture, the stronger the negative phototactic response detected.

Table 2.3.5: Means of duplicate samples obtained from the three light zones of each vessel with χ^2-values corresponding to the phototactic response of *Tisbe*. All χ^2-values are significant at the 5% level.

Vessel	Counts	A	B	C	χ^2-values
1	Adults	2	5	23	25.8
	Copepodids	4	5	26	26.5
	Nauplii	0	0	8	16.0
2	Adults	7	11	23	10.2
	Copepodids	11	23	26	6.3
	Nauplii	7	11	28	16.2
Combined	TOTAL	1.5	2.5	14.3	16.5



(a)



(b)

Figure 2.3.4: Distribution of *Tisbe* as influenced by light level. Italicised superscripts indicate a significant difference ($p<0.05$). a) Illustrates the overall effect of light on the distribution of *Tisbe* individuals. Light = 100 lux, Transition = 30 lux and Shade = 3 lux. b) All demograpahic groups of *Tisbe* investigated: nauplii (NI-NVI), all immature copepodid stages (CI-CV), and adult copepodids (CVI) - exhibited strong negative phototactic behaviour.

2.3.2.2 The effects of salinity and aeration

Tisbe cultures receiving aeration were noticeably more productive than non-aerated cultures. The average final density at the optimal salinities of 25 ‰ and 35 ‰, maintained at 19.5 ±0.1°C, was 7,700 *Tisbe* L^{-1}. The total *Tisbe* population increased from 40 females L^{-1} to a maximum calculated density of 10,200 individuals L^{-1} over the 21-day culture period. The overall average and maximum densities recorded at each sampling event are presented in Table 2.3.6.

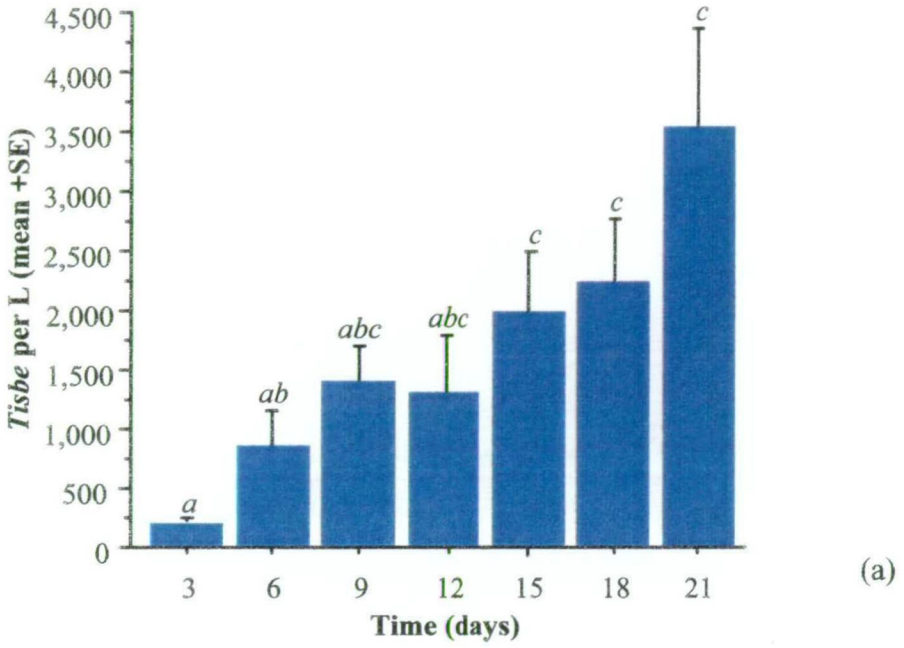
Table 2.3.6: Mean (n=18) and maximum *Tisbe* densities recorded at each of the seven sampling dates presented as individuals 10 mL^{-1} sample collected.

Age of Culture (days)	Mean Density (individuals 10 mL^{-1})	Maximum Density (individuals 10 mL^{-1})
3	2.0	6
6	8.7	47
9	14.1	53
12	13.2	92
15	20.1	79
18	22.6	80
21	35.4	102

Repeated measures ANOVA conducted using square root transformed data revealed that aeration was significantly ($p<0.05$) beneficial to culture productivity with population densities for non-aerated cultures at 530 *Tisbe* L^{-1} compared with 1,730 *Tisbe* L^{-1} for aerated cultures.

The most productive salinity was identified as 35 ‰, yielding an overall mean of 1,445 *Tisbe* L^{-1}, which differed significantly ($p<0.05$) from cultures maintained at both 25 ‰ and 45 ‰ which produced population densities of 1,180 and 695 *Tisbe* L^{-1} respectively (Figure 2.3.5).

Completion of ANOVA for treatment effects at each of the sampling times revealed the beneficial influence of aeration with aerated cultures at 25 ‰ and 35 ‰ significantly ($p<0.05$) more dense *Tisbe* populations than the other four treatments. Short periods of variation from optimal culture conditions did not appear to have an adverse effect on culture productivity, the effect of exposure to salinity regimes either side of the optimum accentuated in the absence of aeration (Figure 2.3.6).



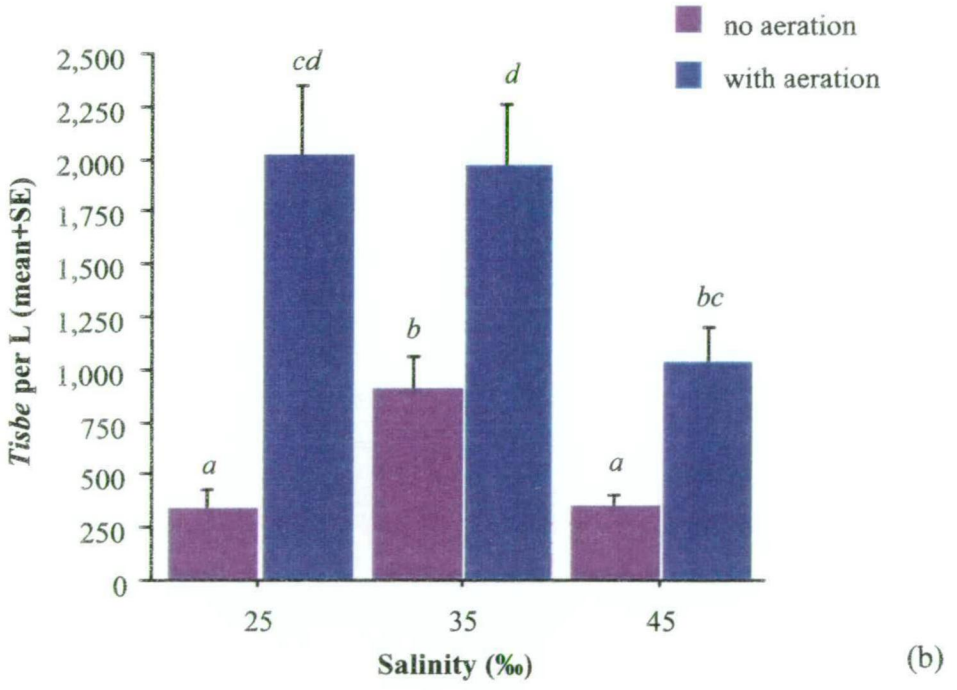


Figure 2.3.5: Influence of salinity (25 ‰, 35 ‰ and 45 ‰) and aeration (with or without) on *Tisbe* culture productivity when maintained at 19.5 °C for 21 days. Italicised superscripts indicate significant differences as determined by ANOVA and Scheffe's multiple means comparison test ($p<0.05$).
a) Increase in *Tisbe* culture population density across all treatment combinations as recorded over the duration of the trial.
b) Repeated measures ANOVA presenting time weighted results for each treatment combination.

After 21 days, salinity, aeration and the interaction between salinity and aeration all exerted significant influences on productivity of *Tisbe* cultures ($p<0.05$). Aerated cultures at 35 ‰ produced *Tisbe* densities of 6,000 individuals L^{-1} that did not differ from the average density of aerated cultures maintained at 25 ‰.

Samples collected on day 3 of the trial (D3) revealed significant salinity effects ($p<0.01$) with the aerated cultures at 45 ‰ yielding the same density as non-aerated cultures at 35 ‰ of 400 *Tisbe* L^{-1}.

No significant treatment effects were evident on day 6 (D6), the maximum mean treatment density being 1,900 *Tisbe* L^{-1} resulting from aerated cultures maintained at 25 ‰.

Day 9 (D9) revealed a high population density in aerated cultures at 45 ‰, which differed significantly ($p<0.001$) from all other culture conditions.

Samples collected on day 12 (D12) revealed no significant differences in *Tisbe* population density between treatments, the highest density of 3,570 *Tisbe* L^{-1} produced by aerated cultures maintained at 35 ‰.

By day 15 (D15) aeration had become a major factor influencing population density, the highest population density of 4,400 *Tisbe* L^{-1} resulting from aerated cultures at 25 ‰. ($p<0.001$).

Both salinity and aeration significantly ($p<0.05$) influenced culture density independently, no significant interaction being recognised by day 18 (D18). Highest *Tisbe* density of 5,100 individuals L^{-1} was recorded for aerated cultures maintained at 25 ‰.

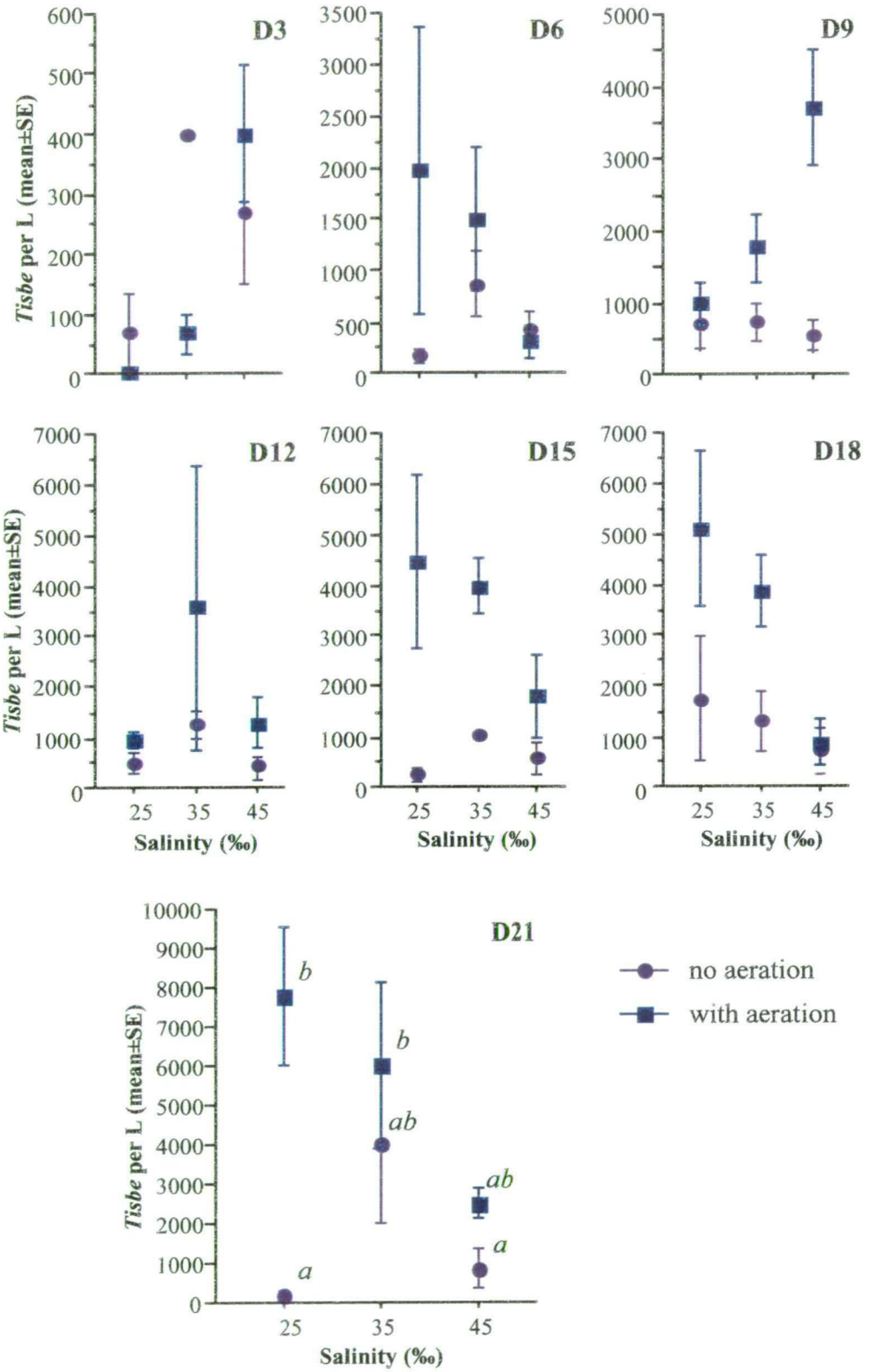


Figure 2.3.6: Influence of salinity and aeration on *Tisbe* culture density over a 21-day period. Treatment salinities tested were 25 ‰, 35 ‰ and 45 ‰. Cultures were maintained at 19.5 °C either with or without aeration. D3 to D21 indicate the day of the trial on which samples were collected. Italicised superscripts indicate significant differences determined between treatments on day 21 ($p<0.05$).

2.3.2.3 The effect of water exchange

Water exchanges, conducted as described in the material and methods section, appear beneficial to *Tisbe* culture productivity, the maximum culture density equivalent to 1,680 *Tisbe* L^{-1} recorded for cultures receiving a once weekly water exchange. Cultures receiving more frequent water exchanges exhibited reduced variation in densities between replicates (Table 2.3.7). However, cultures receiving a once weekly water exchange exhibited the most consistent population composition and the greatest proportion of nauplii.

The temperature range experienced over the trial was 18.5 °C to 20.5 °C with a mean of 19.5 °C ±0.1°C. Salinity averaged 35 ‰ across all cultures varying between 33 ‰ and 37 ‰ over the duration of the trial. Aeration was checked daily and maintained at a moderate rate of 350 mL hr^{-1}.

Table 2.3.7: Population densities and percentage composition (mean ± standard error) for *Tisbe* cultures receiving water exchanges of varying frequency.

Exchange Frequency ($week^{-1}$)	Nauplii %	Copepodids %	Ovigerous Females %	Population Density (*Tisbe* L^{-1})	Range (*Tisbe* L^{-1})
0	26.8 ±3.6	67.7 ±2.1	5.4 ±1.5	1,440 ±320	950 - 2,060
1	37.3 ±2.5	59.6 ±2.2	3.1 ±0.8	1,680 ±160	860 -2,280
2	31.2 ±7.5	64.4 ±7.1	4.4 ±1.8	1,220 ±130	1,080 -1,360
7	25.6 ±5.0	71.3 ±3.7	3.2 ±1.5	1,010 ±140	1,010 -1, 020

2.3.2.4 The effects of temperature and salinity

Temperature exerted a greater influence than salinity on *Tisbe* population density, the most productive cultures being those maintained at 20 °C (720 - 955 *Tisbe* L^{-1}). Population densities of cultures maintained at 20 °C across all salinities investigated were significantly different ($p<0.05$) from all other treatments (Figure 2.3.7). At each temperature, cultures maintained at salinities between 25 ‰ and 35 ‰ were consistently the most productive.

The total number of *Tisbe* individuals developing under the various temperature and salinity combinations resulted in parabolic population growth responses. The influence of salinity on culture productivity was significant at four of the five temperatures investigated, excluding those maintained at 20 °C (Figure 2.3.7).

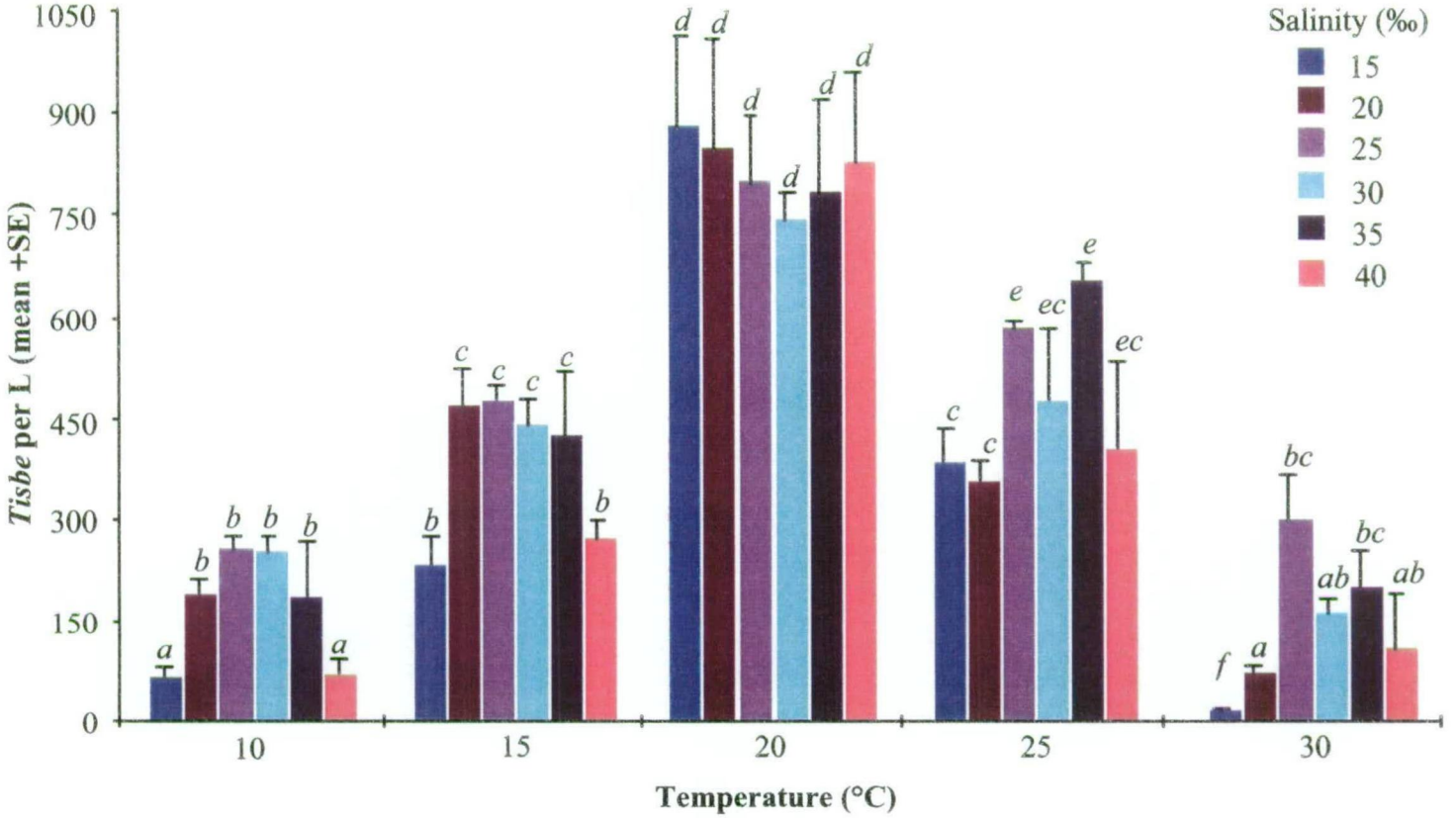


Figure 2.3.7: Effect of salinity and temperature on *Tisbe* productivity as indicated by the total number of individuals developing over nine days from an initial inoculum of five ovigerous females. Italicised superscripts indicate significant differences ($p<0.05$) identified by ANOVA and Scheffe's method for comparison of means.

Temperature and salinity effects were independent with no evidence of an interaction between temperature and salinity on the total number of *Tisbe* individuals.

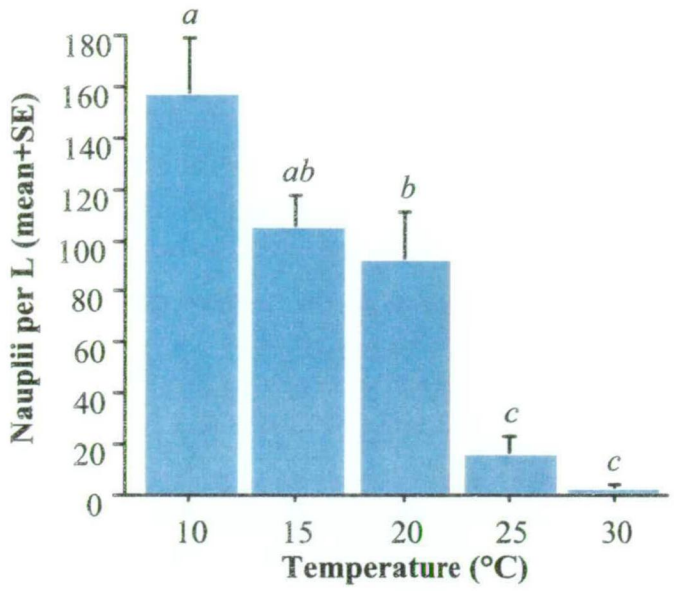
Temperature was observed to exert a significant influence on the proportion of naupliar and copepodid stages developing in cultures (p <0.0001). Nauplii comprised 90% of the population at 10 °C, 28% at 15°C, 10% at 20 °C, 4% at 25 °C and 2% at 30 °C. Copepodids exhibited the complimentary trend in percent composition. The proportion of copepodids at 10, 15 and 20 °C all being significantly different from each other and significantly greater than the proportion recorded at 25 and 30 °C, which were statistically similar (p<0.05).

Nauplii were most numerous at 10 °C, although not significantly different in abundance from those at 15 °C (Figure 2.3.8a). However copepodid numbers exhibited a parabolic response in abundance between temperatures, all means being significantly different from each other (Figure 2.3.8b).

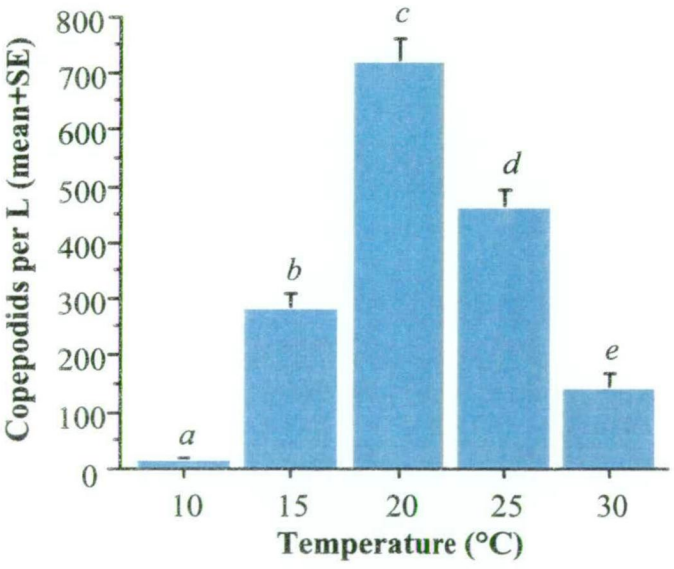
Sex ratio was strongly influenced by temperature (p<0.001) with salinity having no significant effect over the range from 15 to 45 ‰ (Table 2.3.8). No males were found in the samples taken from the cultures maintained at 10 °C and 15°C, the mature females present in the samples being the original females used for the inoculum. The lower temperatures slowed *Tisbe* development so that nine days was insufficient time for nauplii to develop through to maturity. A sex ratio of 3.5 females to each male was expressed by *Tisbe* cultures at 20 °C differing significantly from both those of cultures maintained at 25 °C and 30 °C which were statistically not different, covering a range from 0.6 to 4.0.

The influence of temperature and salinity on the net rate of reproduction (R_0) closely followed the pattern exhibited in response to temperature and salinity on total numbers. Only cultures at 20 °C and 25 °C exhibited average R_0 greater than 1 (Table 2.3.8).

The environmental parameters experienced by *Tisbe* cultured under the various temperature and salinity regimes differed significantly as would be expected. Temperatures and salinities were significantly different (p<0.01) (Appendix A, Tables A5.1.1 and A5.1.2), with no interaction evident between the influence of temperature and salinity on dissolved oxygen levels measured in $mgO_2\ L^{-1}$ or as percent dissolved oxygen. Dissolved oxygen ($mgO_2\ L^{-1}$) was significantly different (p<0.05) between temperatures exhibiting a decline with increasing temperature (Appendix A, Table A5.1.3). The pH of cultures also varied, pH levels of cultures increasing with increasing salinity consistently across all temperature treatments (Appendix A, Table A5.1.4).



(a)



(b)

Figure 2.3.8: Effect of temperature on *Tisbe* culture population composition observed after nine days exposure to six salinities over the range of 15 to 35 ‰ at the five temperatures 10, 15, 20, 25 and 30 °C. Italicised superscripts indicate significant differences as determined by ANOVA of square root transformed data in conjunction with Scheffe's multiple means comparison ($p<0.05$).

(a) abundance of nauplii.

(b) abundance of copepodids.

Table 2.3.8: Summary data for sex ratio (female CVI: male CVI) and net rate of reproduction (*Ro*) estimates for *Tisbe* cultures maintained at five temperatures for nine days.

Treatment (°C)	Sex Ratio (mean ±SE)	Sex Ratio (range)	R_0 (mean ±SE)	R_0 (range)
10	no males	-	0.5 ±0.07	0.0 – 1.4
15	no males	-	0.8 ±0.07	0.4 – 1.8
20	3.5 ±0.32	1.1 – 6.3	2.5 ±0.24	0.4 – 5.6
25	1.8 ±0.15	0.6 – 4.0	2.2 ±0.31	0.4 – 7.0
30	0.9 ±0.15	0.0 – 3.0	0.9 ±0.14	0.0 – 2.8

The trends exhibited by the combined influences of temperature, salinity, dissolved oxygen and pH levels were not reflected in the relative productivities recorded for the cultures developing under the combinations of temperature and salinity investigated.

2.3.2.5 The effect of diet

Diet exerted a significant influence on the total number of *Tisbe* developing in culture (Figure 2.3.9), the size of adult females, and the number and the diameter of eggs carried by ovigerous females. The most dense population of 4,220 individuals L^{-1} was fed the mixture of three microalgae. All fed cultures were statistically similar in terms of *Tisbe* numbers, culture densities only differing significantly from those of unfed cultures. Total number of copepodids (CI through CVI all inclusive) exhibited the same trend with no significant differences between diets in terms of the number of nauplii counted (Table 2.3.9). Ovigerous *Tisbe* were most numerous in the microalgal-fed cultures which differed significantly from those fed the fish crumble and the unfed treatments.

Net reproductive rate (R_0) differed significantly between treatments with microalgal-based diets exhibiting R_0 in the order of three to twenty times greater than fish crumble diets and unfed *Tisbe* culture populations respectively (Table 2.3.10). The sex ratio (female CVI: male CVI) did not differ between diet treatments, the overall mean being 1.12 ±0.58 (SE, n=30).

Prosome length and width of females in addition to egg diameter and the number of eggs per eggs sac were influenced by diet. The largest females were produced on *Nitzschia* diets, however these females were only larger than females developing in cultures fed the mixed algal and the fish crumble diets (Table 2.3.11). The largest egg sacs, containing the smallest diameter eggs, were produced by females fed a diet combining all algal species with the fish crumble (Table 2.3.12).

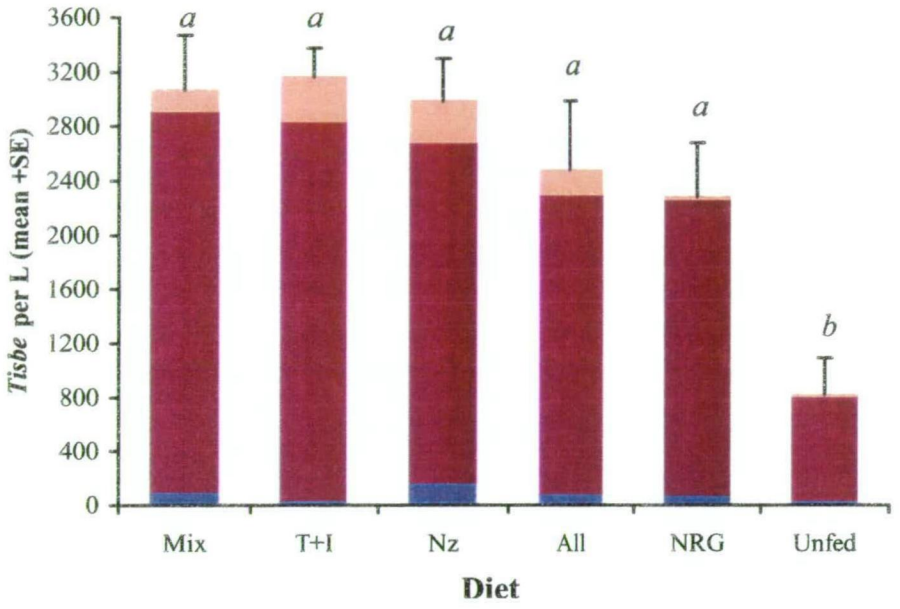


Figure 2.3.9: Influence of six diets on *Tisbe* population density and composition when maintained at 20 °C and 37 ‰ for 14 days. Each column represents the total population density (mean +standard error) with each colour representing the component demographic groups: ▪ ovigerous *Tisbe*, ▪ copepodids CI through CVI excluding ovigerous *Tisbe*, and ▪ nauplius stages NI through NVI. Italicised superscripts indicate significant differences at $p<0.001$. *Mix* – mixture of *Isochrysis*, *Nitzschia* and *Tetraselmis*; T+I - *Tetraselmis* and *Isochrysis*; Nz - *Nitzschia*; All - the three microalgae plus fish crumble; NRG - fish crumble; Unfed - filtered seawater.

Table 2.3.9: Demographic composition of *Tisbe* cultures fed six diets and maintained at 20 °C and 37 ‰ for 14 days. # Copepodid numbers (CI through CVI) reported exclude ovigerous *Tisbe*. Mix – mixture of *Isochrysis*, *Nitzschia* and *Tetraselmis*; T+I - *Tetraselmis* and *Isochrysis*; Nz - *Nitzschia*; All - the three microalgae plus fish crumble; NRG - Lansy® fish crumble; Unfed - filtered seawater.

Diet	Nauplii L^{-1} ($p>0.05$)	Copepodids L^{-1}#	$p<0.05$	Ovigerous *Tisbe* L^{-1}	$p<0.05$
Mix	102.0 ±38.7	2814 ±412	*a*	146.8 ±0.68	*ab*
T+I	40.0 ±12.2	2800 ±216	*a*	318.0 ±25.2	*a*
Nz	169.2 ±95.2	2519 ±333	*a*	296.4 ±25.3	*a*
All	85.6 ±43.9	2213 ±490	*a*	179.6 ±4.53	*a*
NRG	78.0 ±32.4	2187 ±383	*ab*	12.4 ±20.5	*b*
Unfed	40.0 ±20.8	769 ±61.0	*b*	3.2 ±23.0	*b*

Table 2.3.10: Average net reproductive rate (R_0) and sex ratios (female CVI: male CVI) exhibited by *Tisbe* fed six different diets (mean ± standard error). Mix – mixture of *Isochrysis*, *Nitzschia* and *Tetraselmis*; T+I - *Tetraselmis* and *Isochrysis*; Nz - *Nitzschia*; All - the three microalgae plus fish crumble; NRG - fish crumble; Unfed - filtered seawater.

Diet	R_0 (mean ±SE)	$p<0.05$	Sex Ratio (mean ±SE)
Mix	35.4 ±6.32	*a*	0.8 ±0.10
T+I	51.5 ±3.03	*a*	1.1 ±0.11
Nz	42.9 ±7.27	*a*	1.2 ±0.21
All	34.8 ±7.77	*ab*	1.1 ±0.13
NRG	13.8 ±3.08	*bc*	1.2 ±0.14
Unfed	2.68 ±0.26	*c*	1.3 ±0.10

Table 2.3.11: Prosome length and width (mean ± standard error) recorded for adult female *Tisbe* as influenced by diet (mean ± standard error). Mix – mixture of *Isochrysis*, *Nitzschia* and *Tetraselmis*; T+I - *Tetraselmis* and *Isochrysis*; Nz - *Nitzschia*; All - the three microalgae plus fish crumble; NRG - fish crumble; Unfed - filtered seawater.

Diet	Length (µm)	n	$p<0.05$	Width (µm)	n	$p<0.05$
Mix	265 ±2.9	18	*bc*	338 ±6.7	18	*bc*
T+I	267 ±3.2	18	*abc*	342 ±5.2	18	*abc*
Nz	281 ±3.7	18	*a*	336 ±5.5	18	*a*
All	277 ±3.5	18	*abc*	343 ±4.1	17	*abc*
NRG	259 ±5.9	13	*bc*	300 ±9.6	18	*bc*
Unfed	278 ±9.4	17	*ab*	310 ±10.6	17	*ab*

Table 2.3.12: The average number of eggs per egg sac and egg diameters recorded for ovigerous *Tisbe* as influenced by diet (mean ± standard error). Mix – mixture of *Isochrysis*, *Nitzschia* and *Tetraselmis*; T+I - *Tetraselmis* and *Isochrysis*; Nz - *Nitzschia*; All - the three microalgae plus fish crumble; NRG - fish crumble; Unfed - filtered seawater.

Diet	Number of Eggs per Egg Sac	n	$p<0.05$	Egg Diameter (µm)	n	$p<0.05$
Mix	73.0 ±5.04	18	*b*	51.9 ±0.22	180	*ab*
T+I	72.7 ±3.61	18	*b*	54.4 ±0.21	180	*c*
Nz	85.9 ±3.65	18	*bc*	52.8 ±0.21	180	*b*
All	98.5 ±5.97	16	*c*	51.4 ±0.24	161	*a*
NRG	70.5 ±9.37	10	*a*	51.8 ±0.47	100	*a*
Unfed	65.3 ±12.4	4	*a*	54.4 ±0.82	40	*c*

Mean culture temperature was 19.9 ±0.02°C (range from 18.9 °C to 20.2 °C) across treatments. Dissolved oxygen levels (mg O_2 L^{-1}) varied significantly between diet treatments ($p<0.01$) (Appendix A, Table A5.2.1), cultures fed diets containing *Nitzschia* and Lansy® fish crumble exhibiting lower levels than those cultures fed *Tetraselmis* and *Isochrysis*.

At the time the trial was terminated, salinity and pH were found to differ significantly between treatments by ANOVA. However Scheffe's multiple means comparison found only the artificial fish crumble fed cultures to differ from the combined algal plus fish crumble diet by 1.25 ‰. All other diet combinations had similar salinity levels, exhibiting a mean culture salinity of 37.5 ±0.11 ‰

associated with a range from 35.5 ‰ to 39.0 ‰ (Appendix A, Table A5.2.2). Mean pH levels recorded for each treatment differed by 0.42 pH units (7.48 and 7.90), the overall range in values recorded differing only by 0.53 pH units (Appendix A, Table A5.2.3). Significant differences separated unfed cultures and those receiving fish crumble from algae fed cultures. Light levels were found not to differ between treatments, 200 lux the average light intensity recorded (range from 63 to 355 lux).

2.3.3 Larval feeding trials with *Tisbe*

2.3.3.1 Initial feeding trials

Trial 1.1 – The effect of flounder age on the ingestion of Tisbe *life stages*

5 dph flounder

The feeding incidence of flounder larvae first feeding (5 dph) was significantly influenced by live food type (Figure 2.3.10); 89% of flounder larvae offered *Tisbe* nauplii were found to have *Tisbe* nauplii in their gut compared to 29% of the larvae presented rotifers. The flounder presented a mixture of *Tisbe* nauplii and rotifers yielded an overall feeding incidence of 62%. Of the larvae feeding, 43% ingested *Tisbe* nauplii, 7% rotifers, and 50% a mixture of both.

Flounder presented with a single prey type ingested on average per larvae 29 ±6.4 rotifers and 89 ±4.0 *Tisbe* nauplii. When presented a mixed prey diet the first feeding larvae ingested 63 ±8.7 prey larva^{-1}. The ratio of *Tisbe* nauplii to rotifers observed in the gut contents of the larvae ingesting both live food types averaged 4.2 nauplii: 1 rotifer. The unfed control larvae were all found to have an empty digestive tract.

The selectivity index (α) shows that at 5 dph flounder exhibited positive selection for *Tisbe* nauplii with α values greater than 0.5 (α = 0.82, t = 0.86, df = 24) over rotifers (α = 0.18, t = -1.83, df = 24), although the result was not statistically significant (p = 0.039).

The sample of 5 dph flounder larvae exhibited a mean length of 2.74 mm (SE=0.061, n=14), an average width of 185 µm (SE=0.003, n=14) and jaw length of 306 µm (SE=0.006, n=14).

8 dph flounder

The second trial used flounder larvae from the same batch reared on rotifers for a period of three days. The sample of 8 dph larvae exhibited a mean standard length of 3.25 mm (SE=0.061, n=8), an average body width of 294 µm (SE=1.5, n=8) and lower jaw length of 407 µm (SE=1.1, n=8).

The 19% larger and more active 8 dph flounder larvae exhibited similar feeding success rates on all the live food diets presented (Figure 2.3.11). Larvae exposed

to rotifers exhibited a 100% feeding rate, those fed *Tisbe* 85% feeding rate, with only 8% of larvae exposed to *Artemia* feeding successfully. The mean number of prey ingested was 16.0 ±1.95 *Tisbe* larva^{-1}, 14.9 ±4.10 rotifers larva^{-1}, and 0.2 ±0.20 *Artemia* larva^{-1} (SE, n=15 for all data). The unfed control larvae were all found to have an empty digestive tract.

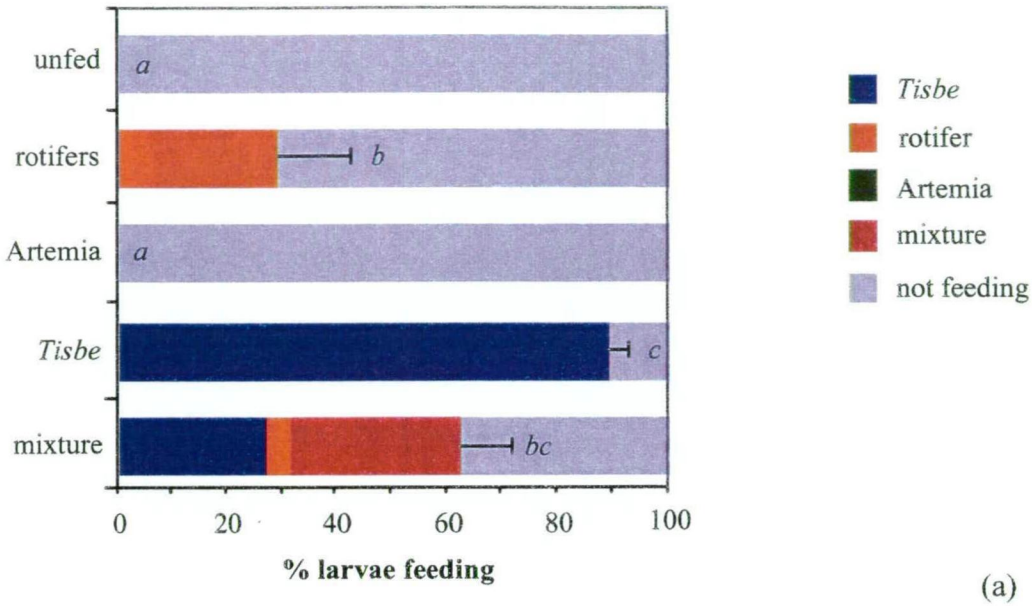
Of the larvae that ingested a mixed diet it should be noted that 30% of the larvae in the mixed prey treatment ingested both copepods and rotifers in comparison to 8% which ingested only copepods and 25% which ingested only rotifers. When larvae were presented a single prey type they ingested on average per larvae 15 rotifers, 15 copepods and 1 *Artemia*. However, those larvae offered a mixture of three prey ingested an average per larvae of 8 rotifers, 1 copepod and less than one *Artemia*.

The selectivity index (α) shows that at 8 dph flounder exhibited significant positive selection 3 for rotifers with α values greater than 0. (α = 0.76, t = 5.58, df = 37) over *Tisbe* nauplii (α = 0.23, t = 3.04, df = 37) and *Artemia* (α = 0.02, t = -738, df = 37) when the three live foods were offered simultaneously. *Tisbe* nauplii were in turn selected in preference to *Artemia* (p<0.003).

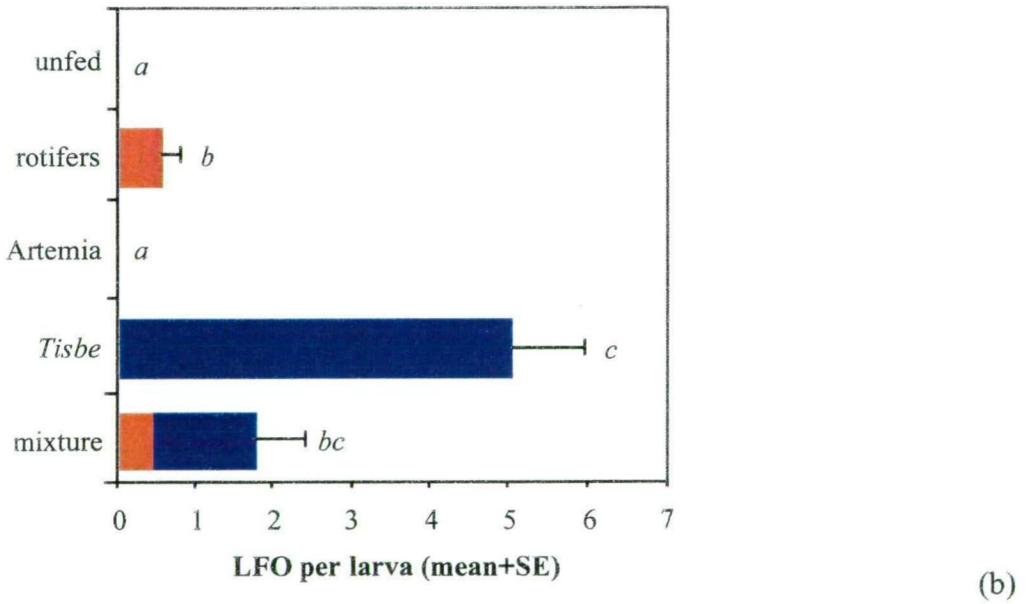
22 dph flounder

The flounder used in this trial had been reared on a combined diet of rotifers and *Artemia* since the trial with 8 dph larvae. The 22 dph larvae exhibited a mean standard length of 5.50 mm (SE=0.157, n=8), an average body width of 1.15 mm (SE=0.061, n=8) and lower jaw length of 788 µm (SE=0.020, n=8).

At 22 dph all larvae presented with live foods were found to have food items in their gut. The unfed control larvae were all found to have an empty digestive tract. The 22 dph flounder consumed an average of 70 rotifers larva^{-1}, 15 *Tisbe* copepodids larva^{-1} or 23 *Artemia* larva^{-1} (Figure 2.3.12). A mixed prey treatment was not offered.



(a)

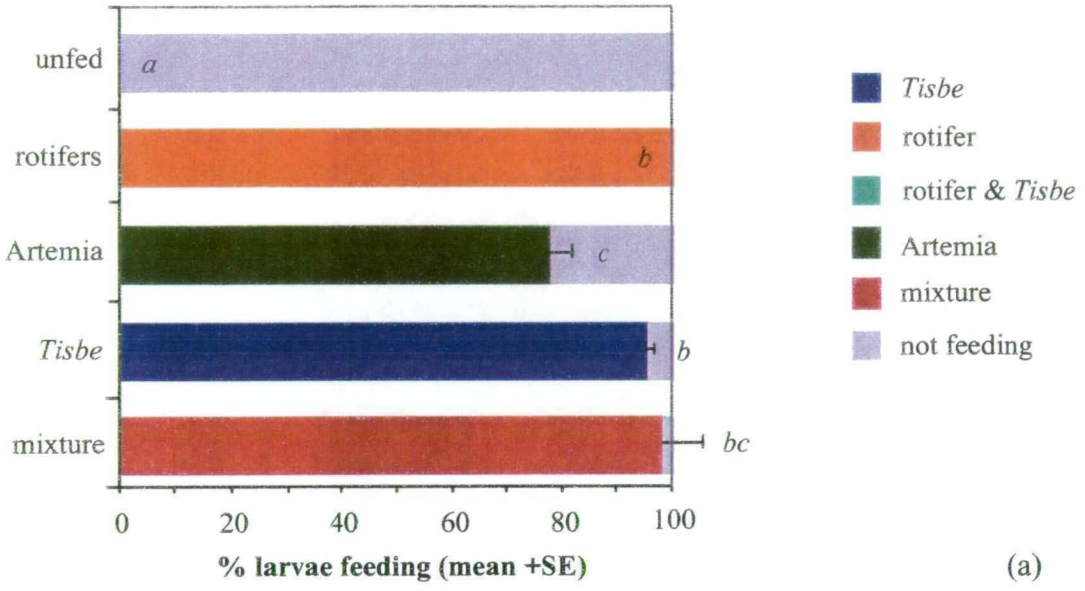


(b)

Figure 2.3.10: Preferences of 5 day post-hatch flounder when presented with four diets: rotifers, *Artemia*, *Tisbe* nauplii, and a mixture of the three at a final density of 10 live feed organisms (LFO) mL^{-1}. An unfed control was also included. Italicised superscripts indicate significant differences ($p<0.05$) identified by the Kruskal-Wallis *k*-sample test and Tukey's multiple means comparison.

a) The proportion of larvae feeding (mean +SE) when presented each of the diets, and

b) the mean number of LFO (mean +SE) recorded from the gut contents of feeding larvae.



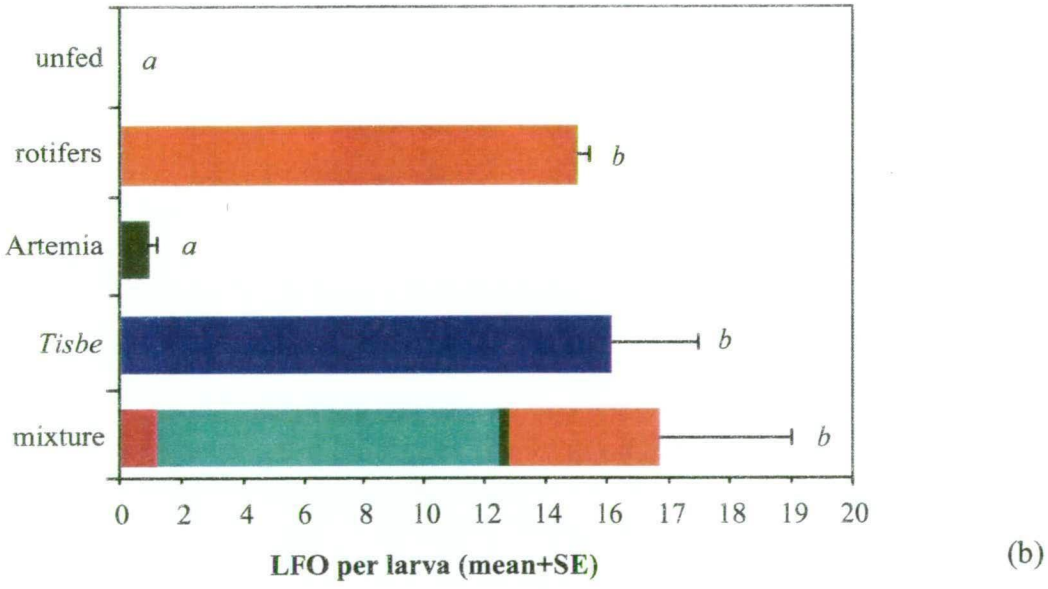
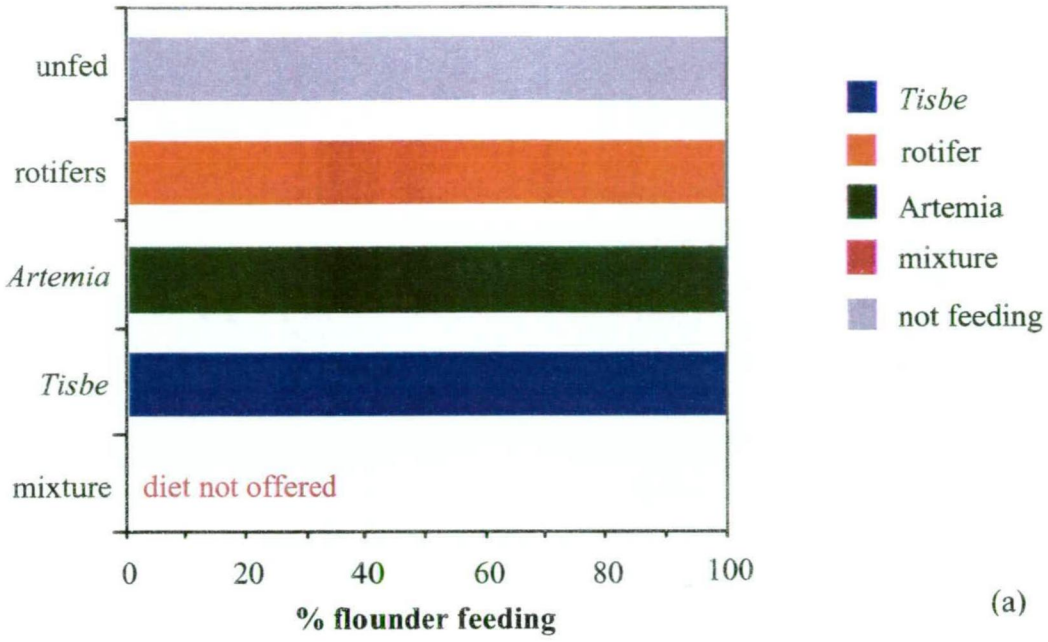


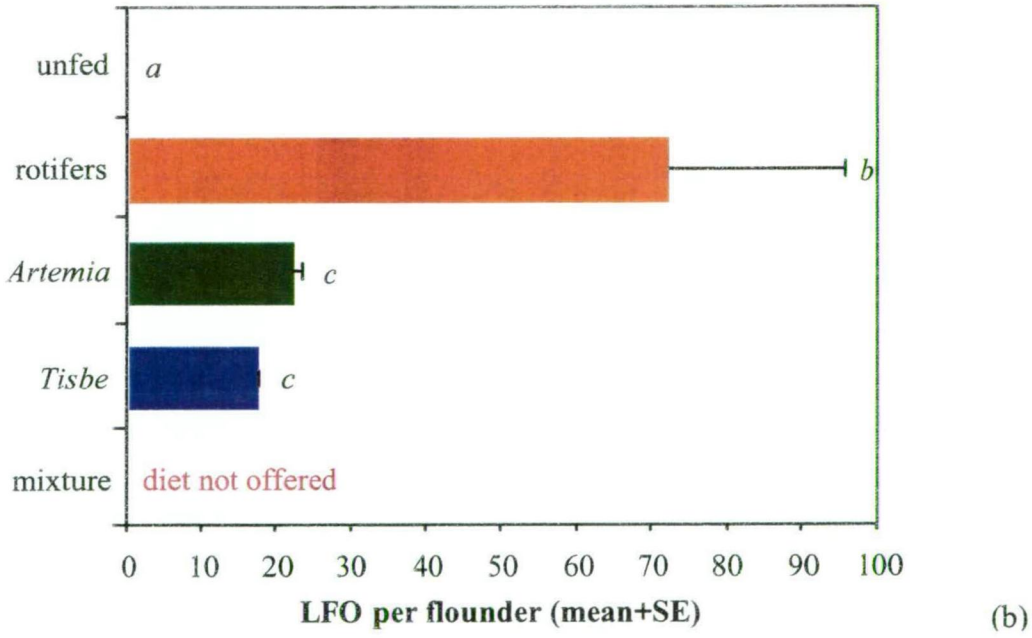
Figure 2.3.11: Preferences of 8 day post-hatch flounder larvae when presented with four diets: rotifers, *Artemia*, *Tisbe* nauplii, and a mixture of the three at a final density of 10 live food organisms (LFO) mL^{-1}. An unfed control was also included. Italicised superscripts indicate significant differences ($p<0.05$) identified by the Kruskal-Wallis *k*-sample test and Tukey's multiple means comparison.

a) The proportion of larvae feeding (mean +SE) when presented each of the diets, and

b) the mean number of LFO (mean +SE) recorded in the gut contents of feeding larvae.



(a)



(b)

Figure 2.3.12: Preferences of 22 day post-hatch flounder larvae when presented with four diets: rotifers, *Artemia* and *Tisbe* nauplii at a final density of 10 live food organisms (LFO) mL^{-1}. An unfed control was also included. Italicised superscripts indicate significant differences ($p<0.05$) identified by the Kruskal-Wallis *k*-sample test and Tukey's multiple means comparison.
a) The proportion of larvae feeding (mean +SE) when presented each of the diets, and
b) the mean number of LFO (mean +SE) recorded in the gut contents of feeding larvae.

Trial 1.2 – Larval feeding incidence as influenced by flounder age and live food type

The 8 dph flounder exhibited a preference for rotifers (Figure 2.3.13a) with an average 14 ±0.7 rotifers (SE, n=26) in their gut contents compared to 2 ±0.4 *Tisbe* nauplii (SE, n=20) and 0.1 ±0.06 *Artemia* nauplii (SE, n=23).

Flounder at 8 dph exposed to *Tisbe* exhibited a higher feeding incidence than those exposed to rotifers (Table 2.3.13) the lowest feeding incidence recorded for *Artemia*-fed flounder larvae.

In contrast, 26 dph flounder exhibited at 100% feeding incidence when exposed to both rotifers and *Tisbe* copepodids, but only 45% when exposed to *Artemia* despite being familiar with the live food (Table 2.3.14). The quantities of each live food recorded from the gut contents of the feeding larvae increased markedly above those recorded for the 8 dph larvae (Figure 2.3.13b).

The determination of the number of individual rotifers from the gut contents of 26 dph larvae was more difficult than with 8 dph larvae as a consequence of the greater digestive action on live foods. Estimates of rotifer numbers were achieved by counting the residual rotifer mastax yielding a mean value of 73.8 per 26 dph larvae, 5 times that recorded from 8 dph flounder.

Missing fish may have been mortalities consumed either by ciliates (observed in the aquaria after the trial, which are assumed to have been introduced with the live foods or larvae), *Artemia* nauplii or copepodid stages present in the treatments.

Table 2.3.13: The percentage of the fifteen 8 day post-hatch flounder larvae feeding, the number of mortalities and the number of larvae missing in the 350 mL mini-aquarium system as influenced by the type of live food presented.

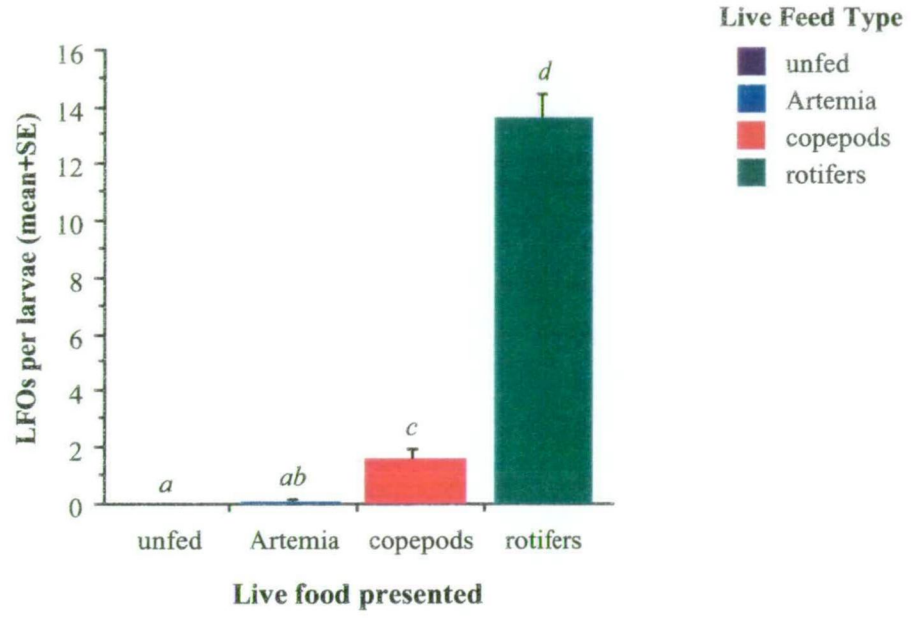
Treatment	Larvae feeding	Larvae not feeding	Larval mortalities	Larvae missing
Control	0%	100%	3	0
Rotifers	87%	13%	0	0
Tisbe	100%	0%	4	3
Artemia	38%	63%	1	4

Table 2.3.14: The percentage of the fifteen 26 day post-hatch flounder larvae feeding, the number of mortalities and the number of larvae missing in the 350 mL mini-aquarium system as influenced by the type of live food presented.

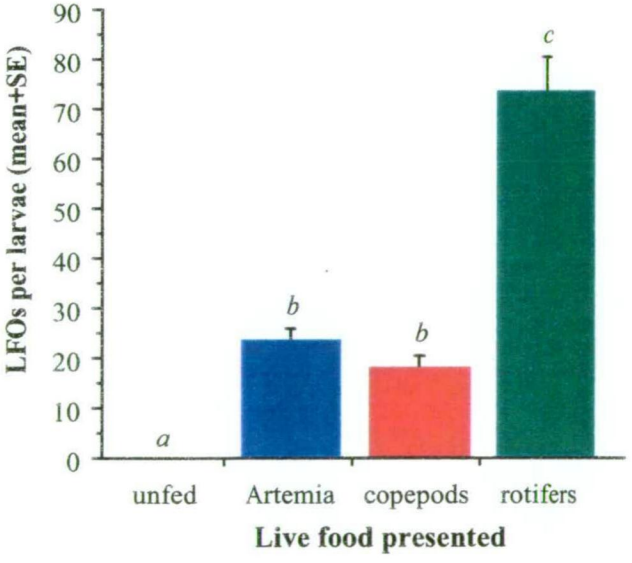
Treatment	Larvae feeding	Larvae not feeding	Larval mortalities	Larvae missing
Control	0%	100%	1	0
Rotifers	100%	0%	1	2
Tisbe	100%	0%	0	1
Artemia	45%	55%	1	3

The copepod diet for 26 dph larvae consisted of copepodid stages CII to CVI of *Tisbe*, which are much larger than rotifers: 445-920 µm in length compared to 220-250 µm in length respectively. The mean number of *Tisbe* copepodids recorded from 26 dph larvae was 18 ±2.7 (SE, n=20), eleven times the number of *Tisbe* nauplii recorded from 8 dph larvae 1.6 ±0.10 (SE, n=20).

Similarly, with 26 dph larvae exposed to *Artemia* the mean number recorded was 23.5 ±2.2 larva^{-1} (SE, n=20), 260 times that recorded for 8 dph larvae 0.08 ±0.03 larva^{-1} (SE, n=20).



(a)



(b)

Figure 2.3.13: Effect of live food type on the gut contents of a) 8 day post-hatch (dph) and b) 26 dph flounder larvae when presented with one of three live food combinations: *Artemia*, *Tisbe* or rotifers at a density of 10 mL^{-1}. An unfed control was included. Italicised superscripts indicate significant differences revealed by ANOVA and identified by Scheffe's multiple means comparison test.

2.3.3.2 Trials with first feeding larvae

Trial 2.1 – Effects of feeding duration and larval preservation on larval gut content retention

The numbers of rotifers identified in the gut contents from fresh and preserved flounder larvae at the onset of exogenous feeding were similar ($p>0.05$, Figure 2.3.14a). Feeding duration significantly influenced the number of live food organisms recorded from the gut contents of the flounder ($p<0.05$, Figure 2.3.14b).

Extended exposure of 5 dph first feeding flounder to rotifers resulted in higher rotifer counts being recorded (Figure 2.3.15a). Analysis of data including the gut contents of all larvae identified three significant time frames. Larvae exposed to rotifers for 1 to 2 hours yielded a mean rotifer count of 0.5 ±0.10 (SE, n=120), increasing to 1.6 ±0.18 (SE, n=120) after 3 to 4 hours exposure. Larvae feeding for 29 to 30 hours are in fact 6 dph larvae, the number of rotifers counted 4 times higher than that recorded for the 5 dph larvae exposed to feeds for 3 to 4 hours, 5.0 ±0.33 rotifers larva^{-1} compared to 1.6 ±0.18 (SE, n=120 for both).

Exclusion of non-feeding flounder from analyses revealed the existence of two time frames corresponding to the first and second days of feeding (Figure 2.3.14b). Feeding incidence at initiation of exogenous feeding yielded a mean of 2.3 ±0.34 rotifers larva^{-1} compared with 5.5 ±0.65 rotifers larva^{-1} recorded from larvae on the second day of feeding. No difference was evident in the number of rotifers recorded from larvae processed fresh or after preservation in 5% formal saline.

The proportion of larvae feeding was observed to increase progressively with an increase in the duration of the trials. Three time frames were apparent in terms of feeding incidence: 1 and 2 hours feeding, 3 and 4 hours feeding, and 29 and 30 hours feeding (Figure 2.3.14c). Over the first four hours of the trial, the proportion of larvae feeding increased from 18.3% to 68.0%. An additional 24 hours larval development time resulted in 96.0% of larvae feeding successfully.

The effect of preservation on feeding incidence was evident only in treatments exposed to feeds for 1 hour (Figure 2.3.14c). The feeding incidence recorded for flounder processed fresh after one hour was ten times greater than from their preserved counterparts: 30 ±15.3% compared with 3.3 ±3.33% (SE, n=3) respectively.

No significant difference was detected in larval dimensions recorded between the twelve different feeding duration and preservation treatment combinations ($p>0.05$). The flounder larvae used in the investigation exhibited mean total length 2.9 ±0.01 mm, average body width of 190 ±0.7 µm and lower jaw length of 326 ±1.0 µm (mean ±SE, n=350).

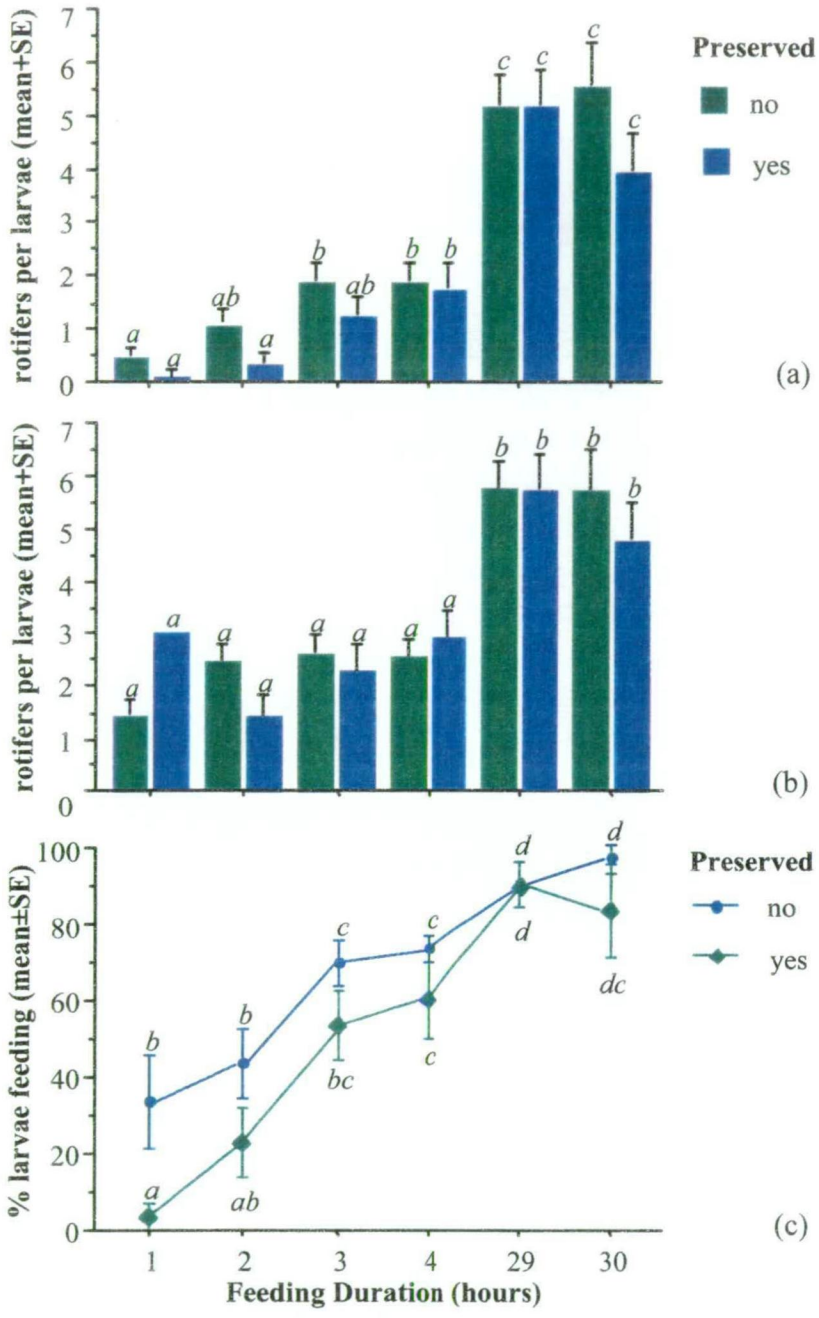


Figure 2.3.14: Effect of preservation and feeding duration on the gut contents of first feeding flounder larvae (5 days post-hatch) exposed to rotifers for 1, 2, 3, 4, 29 or 30 hours. Italicised superscripts indicate significant differences between means as determined by ANOVA of square root transformed data and identified by Scheffe's multiple means comparison ($p<0.05$).

a) The mean number of rotifers per flounder larvae.

b) The number of rotifers per feeding flounder larvae when all larvae with an empty gut excluded.

c) The proportion of flounder larvae feeding, as determined by the presence of material in the larval gut.

Trial 2.2 - Live food preference of first feeding larvae

Flounder larvae exhibited a preference for *Tisbe* nauplii over rotifers (α= 0.79, t= 6.29, p<0.0000). The number of *Tisbe* nauplii counted from larvae presented *Tisbe* in isolation or as a component of the mixture was greater than the number of rotifers observed in larval gut contents from either treatment (p<0.001) (Table 2.3.15).

More *Tisbe* nauplii were consumed by flounder at 5 dph when presented alone (5.0 ±0.60, n=45) than in combination with rotifers (1.3 ±0.23, n=45) (p<0.001). In contrast, a similar number of rotifers were ingested in the presence of *Tisbe* nauplii (0.3 ±0.09, n=45) and when presented alone (0.5 ±16, n=45) (p<0.01).

The dimensions recorded for the 5 dph flounder larvae did not differ between treatments (p>0.05) exhibiting mean total length of 2.9 ±0.01 mm, average width of 167±1.0 µm and mean jaw length of 323 ±1.9 µm (mean ±SE, n=180).

Table 2.3.15: Preferences of 5 dph flounder when presented with rotifers and *Tisbe* nauplii at a final density of 10 mL^{-1}. Mixture indicates rotifers plus *Tisbe* nauplii presented in a 1:1 ratio. Italicised scripts indicate means which are significantly different. # indicates components of mixture treatment attributed to each live food type.

Live food	LFO $larva^{-1}$ (mean ±SE)	Minimum	Maximum	p<0.05
Unfed	0.0 ±0.02	0	1	*a*
Rotifers	0.5 ±0.16	0	5	*ab*
Tisbe	5.0 ±0.60	0	18	*c*
Mixture	1.6 ±0.25	0	6	*b*
#rotifer	#0.3 ±0.03	-	-	-
#*Tisbe*	#1.3 ±0.57	-	-	-

2.3.3.3 Trials with 8 day post-hatch larvae

Trial 3.1 – The effect of live food type & preservation on larval gut content

Consistently higher gut content counts were obtained from preserved 8 dph flounder larvae than for those processed fresh, however the difference was only significant for flounder fed *Tisbe* life stages (p<0.05, Figure 2.3.15).

Tisbe nauplii could be seen moving in the gut of the larvae (as with rotifers occasionally) when viewed under the dissecting microscope. Two larvae had an amorphous substance in their gut contents closely resembling copepod faecal material, possibly ingested during attempts to capture nauplii.

The combined mean number of *Tisbe* nauplii 16.4 ±1.7 larva^{-1} ingested by 8 dph larvae, although slightly higher is not significantly different (p>0.05) from the combined mean number of rotifers ingested (13.4 ±1.2 larva^{-1}) (mean ±SE, n=25). The number of *Artemia* 0.8 ±0.17 larva^{-1} (mean ±SE, n=53) ingested was not significantly different from the unfed control.

Flounder larvae of 8 dph ingested a mean of 9.4 ±0.60 LFO larva^{-1} (mean ±SE, n=50) when presented a mixture of rotifers, *Artemia* and *Tisbe* being significantly different (p<0.05) to the frequency of all other diets offered. When presented a mixed diet, the 8 dph flounder exhibited a preference for rotifers over *Tisbe* nauplii and *Artemia*: 0.2 ±0.09 *Artemia* larva^{-1} and 1.4 ±0.24 *Tisbe* larva^{-1} compared to 8.0 ±0.62 rotifers larva^{-1} (mean ±SE, n=50).

The dimensions of 8 dph flounder did not differ between treatments (p>0.05) exhibiting a mean total length of 3.6 ±0.02 mm and a mean jaw length of 454 ±2.9 µm (mean ±SE, n=255).

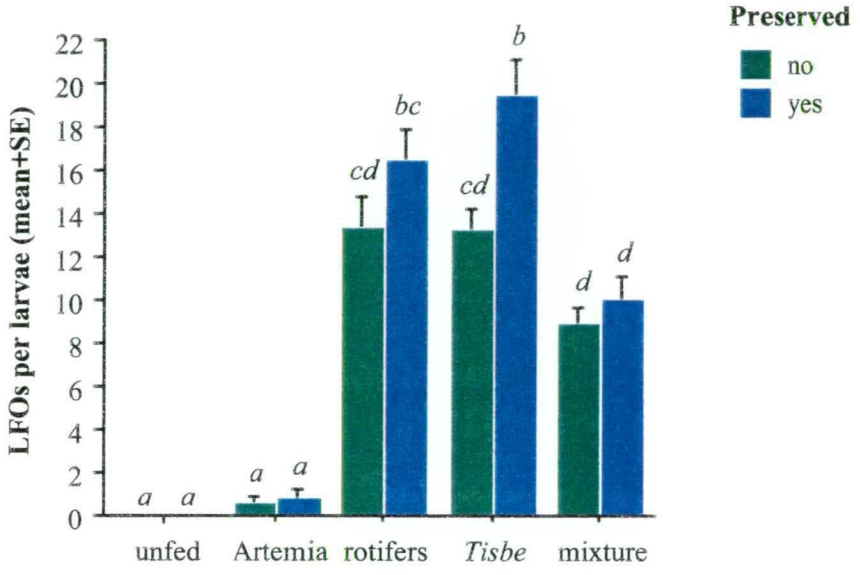


Figure 2.3.15: Effects of preservation and live food type on the number of live food organisms (mean LFO ±SE) found in the gut contents of 8 day post-hatch flounder presented with one of five diets at a final density of 10 mL^{-1}: rotifers, *Artemia*, *Tisbe* nauplii, a mixture of the three, and an unfed control. Italicised superscripts indicate significant differences determined through the use of the non-parametric Kruskal-Wallis *k*-sample analysis and Tukey's multiple means comparison.

Trial 3.2 – The effect of feeding duration on larval gut content

Feeding duration was found to have a significant effect on the gut contents of 8 dph flounder ($p<0.05$, Table 2.3.16). Larvae exposed to live foods for 2 to 5 hours resulted in similar numbers of LFO being counted from the gut contents of feeding larvae: 13.4 ±0.70 LFO larva^{-1} (mean ±SE, n=112). The numbers of prey items counted from gut contents after one hour and six hours exposure to live foods were not significantly different ($p>0.05$) at 8.4 ±0.87 LFO larva^{-1} (SE, n=53).

The mean total length exhibited by the 8 dph flounder used in the investigation was 3.4 ±0.02 mm corresponding to a mean lower jaw length 446 ±2.4 µm (mean ±SE, n=164) and did not differ significantly between treatments ($p>0.05$).

Table 2.3.16: The influence of feeding duration on the gut contents of 8 dph flounder when presented with rotifers at a density of 10 mL^{-1} for either 1, 2, 3, 4, 5 or 6 hours. Italicised scripts indicate significant differences ($p<0.05$) identified by analyses of variances and Scheffe's multiple means comparison test conducted using square root transformed data.

Exposure Time (hours)	rotifers larvae^{-1} (mean ±SE)	n	$p<0.05$
1	8.1 ±1.35	26	*a*
2	14.1 ±1.37	29	*b*
3	12.4 ±1.16	29	*b*
4	13.4 ±1.15	29	*b*
5	14.0 ±1.95	25	*b*
6	8.8 ±1.15	26	*a*

2.3.3.4 Trials with 15 and 25 dph larvae

Flounder from the two age groups exhibited 100% feeding incidence with the exception of the unfed control treatments, which had no material in their digestive tracts. Differences were evident between the two age groups with respect to the number of rotifers and *Artemia* recorded, however not for diets including *Tisbe* copepodids (Figure 2.3.16).

When both age groups were presented *Tisbe* copepodids, similar numbers of prey items were counted from the gut contents of both groups: 5.1 ±1.02 *Tisbe* from 15 dph larvae compared with 5.8 ±0.64 *Tisbe* from 25 dph flounder (mean ±SE, n=30 for all data). Similarly, when presented mixed prey diets, prey item counts from the gut contents of both age groups were the same: 11.0 ±0.94 LFO 15 dph larva^{-1} and 11.1±1.5 LFO 25 dph larva^{-1}.

In contrast, the number of rotifers and *Artemia* recorded from the gut contents of the flounder increased markedly from 8.4 ±1.4 rotifers larva^{-1} and 4.2 ±0.65 *Artemia* larva^{-1} at 15 dph to 16.8 ±2.8 rotifers larva^{-1} and 16.8 ±1.7 *Artemia* larva^{-1} at 25 dph.

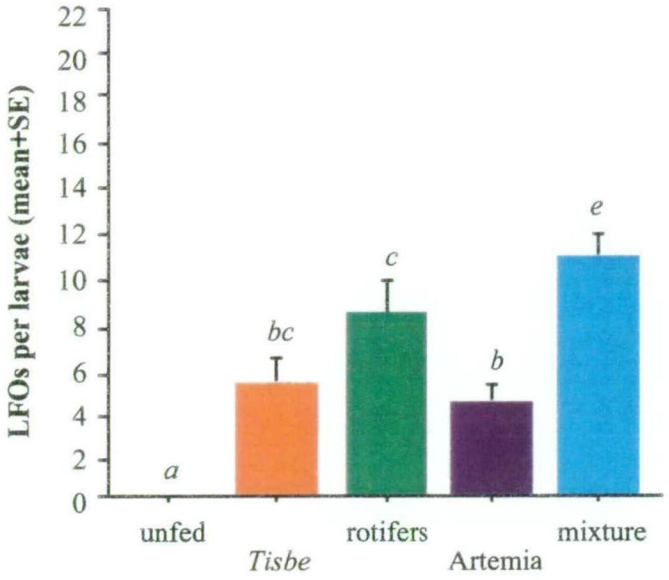
Larvae consumed significantly more LFO when presented with a mixture, than when presented with individual live food diets (Figure 2.3.16a). The 25 dph flounder larvae consumed significantly more rotifers and *Artemia* when presented single species diets, the introduction of *Tisbe* to the mixture resulting in a significant decrease in the mean number of live food individuals recorded (Figure 2.3.16b).

Differences between the two flounder age groups are also evident in the prey composition of the gut contents when presented a mixed LFO diet (Table 2.3.17). The number of rotifers and *Tisbe* consumed decreases coincident with an increase in the number of *Artemia*.

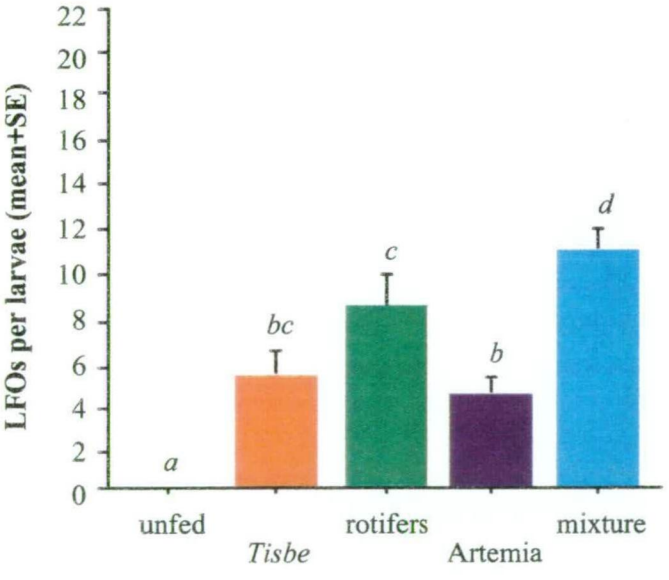
Table 2.3.17: Gut content composition for flounder of 15 dph and 25 dph when presented a mixed diet or rotifers, *Artemia* and *Tisbe* copepodids at a final density of 10 mL^{-1} (n=30).

Diet	LFO larva^{-1} (mean ±SE)	
	15 dph	25 dph
Unfed	0.0 ±0.00	0.0 ±0.00
Rotifers	6.7 ±1.22	3.3 ±1.25
Artemia	0.5 ±0.22	6.0 ±0.85
Tisbe	3.8 ±0.92	1.8 ±0.32
Total	11.0 ±0.94	11.1 ±1.46

The dimensions recorded for the 15 dph and 25 dph flounder larvae did not differ between diet treatments (p>0.05). During the 10 days between trials the flounder had increased in length from 3.9 ±0.21 mm to 5.4 ±0.03 mm, associated with an increase in average width from 0.5 ±0.07 mm to 1.7 ±0.02 mm and mean jaw length from 478 ±3.2 µm to 795 ±4.4 µm (mean ±SE, n=150 for all measurements).



(a)



(b)

Figure 2.3.16: Influence of live food type on the gut contents of a) 15 day post-hatch (dph) and b) 25 dph flounder larvae when presented with *Tisbe* copepodids, rotifers, *Artemia*, or a mixture of the three live food organisms (LFO) at a final density of 10 mL^{-1}. Italicised superscripts indicate significant differences ($p<0.05$) identified by analyses of variances and Scheffe's multiple means comparison conducted using square root transformed data.

2.3.3.5 Live food preference as influenced by larval flounder age

Larval Feeding Preferences

Flounder larvae exhibited a change in preferred live food organism with increasing age progressing from *Tisbe* nauplii to rotifers to *Artemia*.

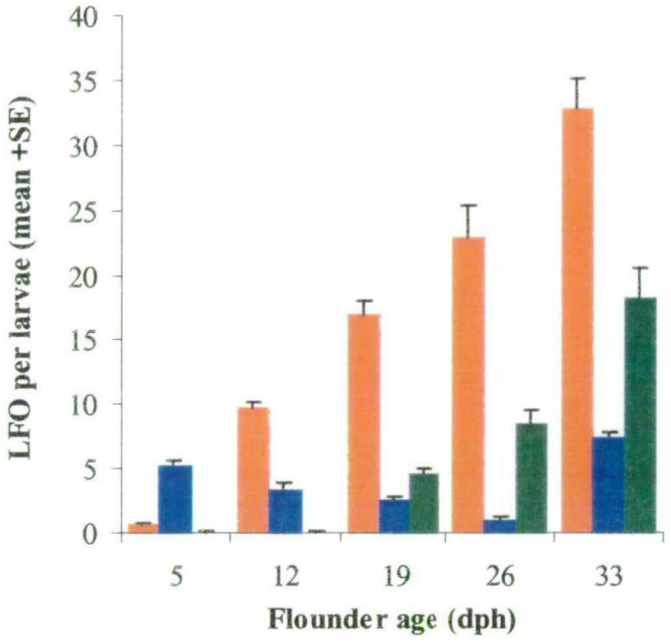
Flounder of 5 dph preferred *Tisbe* nauplii (α 0.79, t=7.85, $p<0.0001$) to rotifers and *Artemia*. Larvae of 12 dph, 19 dph and 26 dph selected rotifers from a mixed diet (α of 0.98, 0.90 and 0.77, corresponding to t values of 8.17, 7.94 and 7.19 respectively, all with $p<0.0001$). At 33 dph flounder selected positively for *Artemia* (α 0.62, t=4.25, $p<0.0001$).

The mean total number of live food organisms (LFO) counted from the gut contents of flounder from the five age classes increased significantly ($p<0.001$) with development time from 1.1 ±0.22 LFO larva^{-1} at first feeding (SE, n=225) to 19.5 ±1.15 LFO larva^{-1} at 33 dph (SE, n=195) (Figure 2.3.17a).

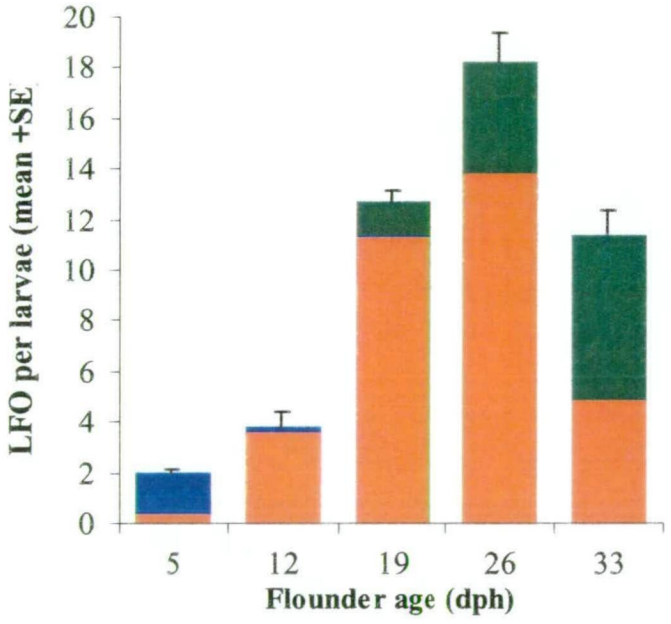
The number of rotifers in the gut increased from 0.5 rotifers larva^{-1} to 32.9 rotifers per 33 dph larva, an overall increase of 6,580% from the onset of exogenous feeding. The number of *Artemia* nauplii recorded increased from 0 *Artemia* larva^{-1} at 12 dph to 4.4 at 19 dph. An average 18.2 *Artemia* nauplii larva^{-1} were consumed by 33 dph: an overall increase of 414% (Appendix A, Table A6.3.1).

The number of *Tisbe* life stages recorded from flounder gut contents fluctuated considerably from one age group to the next. In contrast to the progressive increase in the number of live food organisms observed in the gut contents of flounder presented rotifers and *Artemia*, flounder larvae offered *Tisbe* exhibited a decline in the number of copepod life stages recorded from gut contents: 5 dph larvae were found to have consumed an average 5.2 nauplii, which decreased numerically to 3.2 early stage copepodids larva^{-1}, 2.4 copepodids larva^{-1}, and 1.0 copepodid larva^{-1} from ages 12, 19 and 26 dph. However, flounder at 33 dph consumed the greatest number of *Tisbe* life stages with an average 7.5 copepodids larva^{-1} (Table 2.3.18). The apparent anomaly may be explained by the increasing size of the *Tisbe* life stage targeted by the older larvae.

The results from the mixed diet exhibit similar trends (Figure 2.3.17b). Flounder of all ages continued to ingest *Tisbe* life stages when present in a mixed diet, exhibiting an initial decrease in the number of *Tisbe* targeted before increasing at 33 dph. A strong preference was expressed for rotifers to the exclusion of *Tisbe*, and to a lesser extent *Artemia* by 26 dph.



(a)



(b)

Figure 2.3.17: Gut contents of flounder larvae of five different ages after two hours exposure to live food organisms (LFO) at a final density of 10 mL^{-1}. Italicised superscripts indicate significant differences ($p<0.05$) identified by Kruskal Wallis *k*-sample and Tuckey's multiple means comparison.

(a) Single species diets of ▪ rotifers, ▪ *Artemia* or ▪ *Tisbe*.

(b) Mixed live feed diet comprising equal numbers of ▪ rotifers, ▪ *Artemia* and ▪ *Tisbe* life stages. The relative heights of the bars indicate the total number of LFO $larva^{-1}$.

Larval dimensions

Within each age group (5, 12, 19, 26 and 33 dph), no significant difference was detected in the dimensions of the larvae exposed to different live food diets and the unfed controls. However ANOVA of log transformed length data revealed a highly significant difference ($p<0.0001$) between age groups for standard body length, body width and lower jaw length.

The standard length of the flounder larvae increased 380% in four weeks from 2.9 mm to 6.7 mm (Appendix A, Table A6.3.2), corresponding to a 1080% increase in body width reflecting the flattening of the larval body shape during metamorphosis (Appendix A, Table A6.3.3). The lower jaw length parameter demonstrated an increase of 153% from 5 dph to 26 dph, followed by a 4% decrease from 26 dph to 33 dph (Appendix A, Table A6.3.4).

2.4 Discussion

This study has shown that the Tasmanian species of *Tisbe* conforms to the general harpacticoid life cycle, exhibits tolerance of a wide range of environmental conditions and is easy to maintain in culture. *Tisbe* was well accepted as a live food by flounder larvae. A strong preference was exhibited by first feeding flounder larvae for *Tisbe* nauplii over the traditional rotifers, and as the flounder larvae developed they were observed to ingest increasing larger life stages of *Tisbe.*

2.4.1 Life cycle and demographics

The temperate Australian species of *Tisbe* used for these trials demonstrated life cycle and demographic characteristics consistent with those reported for Northern Hemisphere congeners.

This is the first comprehensive report documenting the life cycle and associated stages of development for the Tasmanian species of *Tisbe.* This investigation identified twelve distinct life stages in the life cycle of *Tisbe* agreeing with the generic harpacticoid life cycle depicted in Wells (1988) and published information for the Northern Hemisphere congeners: *T. cucumariae* (Lopez, 1980) and *T. holothuriae* (Støttrup & Norkser, 1997). The representative lengths of the 12 life stages recorded for *Tisbe* are similar to those published for *T. cucumariae* (Lopez, 1980) (Figure 2.4.1).

Sexual dimorphism is obvious for *Tisbe* from the fifth copepodid stage onwards. Females are 21% larger than males and they exhibit distinctly different antennule morphology. Both antennules of the mature male are geniculate, with no obvious specialisation of the fifth pair of thoracic appendages between genders (Davis, 1984; Huys & Boxshall, 1991).

Distinguishing characteristics for each stage are based on significant length differences, in combination with anatomical differences readily identified under a dissecting microscope (Table 2.4.1). It is important from a technical aquaculture perspective that stages are easily identified to assist with assessment of copepod culture health and selection of suitable copepod stages for feeding to finfish larvae.

The Tasmanian species of *Tisbe* exhibits significant live food potential in terms of food particle size. The size limit of food particles able to be ingested by marine finfish larvae at the onset of feeding has been reported as 100 µm (Nellen, 1985; Watanabe & Kiron, 1994; Iglesias et al., 1994). Combined with the general consensus that prey width is the most important dimensional consideration for ingestion (Shirota, 1970; Ghan & Sprules, 1993; Fernández-Diaz et al., 1994), *Tisbe* possesses at least two nauplius stages which meet these criteria: NI possessing a mean width of 67 µm and NII a width of 78 µm; with corresponding lengths of 80 and 98 µm respectively.

Table 2.4.1: Features useful in differentiating between life stages of *Tisbe*. The length values reported are indicative of relative size differences corresponding to the distance from the rostrum to feruncular setae with descriptions modified from Lopez (1980).

Life Stage	Length (µm)	Identifying Features:
N I	80	▪ dorso-ventrally flattened circular shape ▪ posterior edge rounded with two setae ▪ anus is absent
N II	98	▪ telsonic differentiation commences ▪ posterior slightly elongated ▪ shallow caudal notch between the two setae
N III	136	▪ telsonic articulation appears ▪ two setae either side of caudal notch
N IV	156	▪ 1st pair of maxillule basal buds apparent ▪ broader posteriorly and narrower anteriorly ▪ caudal a little more defined
N V	175	▪ 2nd pair of buds corresponding to first periopods appear ▪ posterior more elongate ▪ caudal notch more defined
N VI	197	▪ 3rd bud pair corresponding to 2nd periopods appear ▪ posterior end almost segmented ▪ 3 caudal seate visible
C I	258	▪ resembles adult form with broader cephalosome with a narrower urosome terminating in caudal furca ▪ 1st pairs of periopods on cephalosome ▪ 2nd pair of periopods on first thoracic segments ▪ urosome comprises single somite
C II	326	▪ 3rd pair of periopods appear on 2nd thoracic segment ▪ urosome has 2 somites
C III	375	▪ 4th pair periopods appear on 3rd thoracic segment ▪ urosome has 3 somites
C IV	430	▪ slender setae represent 5th periopod pair on first urosomal segment ▪ urosome has 4 somites
C V m	540	▪ 5th periopod pair segmented ▪ urosome comprises 5 somites
C V f	540	▪ 5th periopod pair segmented ▪ urosome comprises 5 somites
C VI M	648	▪ antennules 9 segmented, stout and both geniculate ▪ urosomal segments similarly sized, genital somite marginally larger
C VI F	825	▪ antennules 9 segmented, slender and non-geniculate ▪ genital pore comprises 2 fused somites ▪ single egg sac extruded from genital pore

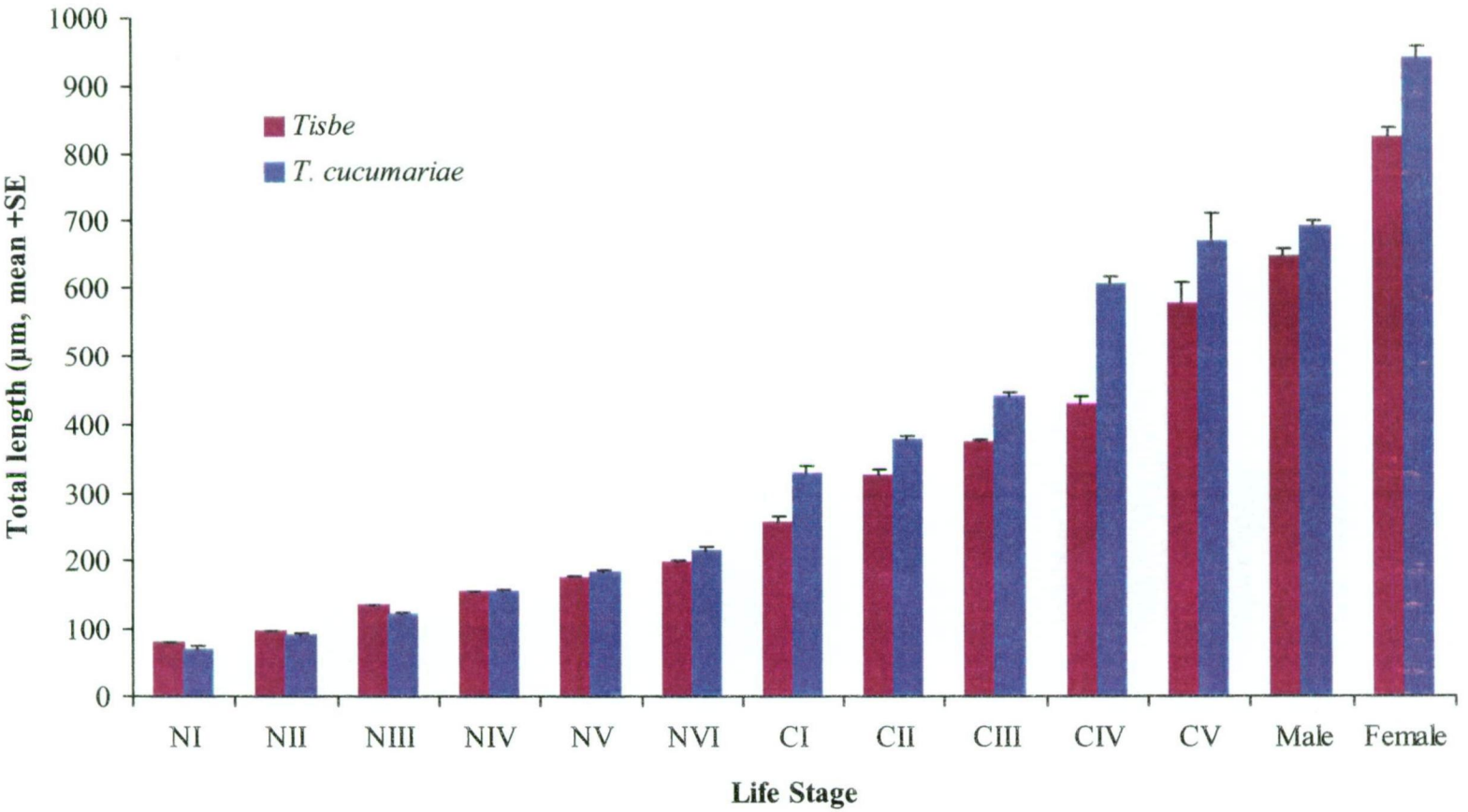


Figure 2.4.1: Comparison of the total length (measured, in µm, from the rostrum to the end of the last urosomal segment excluding the feruncular setae; mean + standard error) of two species of *Tisbe*: the Tasmanian species and the Northern Hemisphere *T. cucumariae* (Lopez, 1980). The six nauplius stages are denoted NI through NVI, the sexually immature copepodid stages CI through CV. Sexual dimorphism is illustarated by the Male and Female distinction for copepodid stage CVI.

Northern Hemisphere representatives of *Tisbe* have demonstrated this potential since as early as 1969 when Barr was able to culture sufficient numbers of nauplii to rear larval fish (Barr, 1969). More recently *T. cucumariae* and *T. holothuriae* have been used successfully as a live food for turbot (Norsker & Støttrup, 1994) and haddock larvae (Nanton & Castell, 1999).

The approximately twelve hour duration observed for *Tisbe* nauplii would mean that live food of an appropriate size would be available to finfish larvae nearing the onset of first feeding for a sufficient exposure time to maximise the chance of successful commencement of exogenous feeding.

The 7 day mean generation time (T) observed for *Tisbe* when maintained at 23 °C is significantly shorter than that reported by Northern Hemisphere studies. Milou & Moraitou-Apostolopoulou (1991a,b) reported a mean generation time (T) of 11.1 days for *Tisbe holothuriae* cultured at 19 °C and 38 ‰. The shorter T observed for the Australian *Tisbe* (in the order of 7 days at 23 °C) may in part be attributed to the higher culture temperature (Uye, 1988; Klein Breteler, 1994; Takahashi & Ohno, 1996).

The net rate of reproduction (R_0) reported for the Tasmanian species (in the order of 10) was significantly lower than the 29.3 reported by Milou & Moraitou-Apostolopoulou (1991a,b). The relatively food poor conditions under which *Tisbe* were maintained in conjunction with the elevated culture temperature were factors contributing to the lower R_0.

The observation by Abu-Rezq et al. (1997) that early egg sacs produced by *Tisbe furcata* females were larger in size with consistently more nauplii hatching than those produced later in life was also evident in culture of the Tasmanian *Tisbe*.

Demographic variable r_m, which characterises population proliferation, was found to vary from 0.304 under best nutritional conditions (Ulva + fryfood) to 0.24 for yeast (Milou & Moraitou-Apostolopoulou, 1991b). The calculation of estimated r_m according to Milou & Moraitou-Apostolopoulou (1991a) for the Tasmanian *Tisbe* yielded comparable values ranging between 0.3 and 0.2.

Difficulties were inherent in the logistics involved in tracking the productivity of a single female and her subsequent progeny individually. The data obtained are more detailed than that required to monitor the productivity and health of cultures maintained as live food for fish larvae.

Aspects of female demographics such as the proportion of females bearing egg sacs and the relative size of the egg sacs may be far better indicators of culture health and productivity. The importance of female egg production to population dynamics is reflected in the use of egg production as a means by which to estimate

field productivity (McLaren & Leonard, 1995; Poulet et al., 1995; Calbet & Alcaraz, 1996).

The trial indicated that the determination of the finer demographic details, although of importance in the determination of ecological budgets, were not of sufficient value to the applied nature of the work to warrant being repeated. A similar amount of effort is required to maintain small volume mass cultures which yield results more representative of those likely to be achieved under mass culture conditions used to produce copepods as live food for larviculture.

2.4.2 Culture of *Tisbe*

The Tasmanian species of *Tisbe* exhibits behaviours which could be used to assist in the harvest of live food for feeding to fish larvae in addition to exhibiting a tolerance of a wide range of environmental conditions. Light, temperature and salinity can be manipulated to maximise culture productivity. The manipulation of diet and culture system further improved the productivity of *Tisbe* cultures.

All twelve stages in the life cycle of *Tisbe* are negatively phototactic, showing a preference for shade over full light. Lopez (1980) reported nauplii of *Tisbe furcata* to be negatively phototactic and to cling to food particles on the bottom of the container. Although lower light levels are preferred by all stages of *Tisbe*, even illumination of the larval rearing tank should assist in maintaining a more homogenous distribution of copepod life stages. To achieve maximum availability of *Tisbe* to larvae, even illumination of larval rearing tanks at the lowest intensity should be maintained without comprising larval predation capabilities.

Light has the potential to be used as a harvesting tool for *Tisbe* with darkened areas of the culture vessel acting as aggregation points from individuals can be siphoned thus reducing the volume of culture media needed to be screened to obtain the desired quantity of feed organisms. Støttrup & Norsker (1997) in the bioreactor used a directed light source to drive copepodids away from the nauplius harvest point. The implied reduced photonegative response of nauplii may in fact be a reflection of the reduced swimming ability of nauplii compared to copepodids.

The most productive temperature was identified as 20 °C, with salinity over the range of 15 to 40 ‰ having no influence on final density after nine days. At temperatures either side of the optimum, salinity was found to influence productivity; salinities outside the range 25 to 35 ‰ resulting in reduced population density after nine days. In Northern Hemisphere research Milou & Milou-Apostolopoulou (1991a) identified 19 °C and 38 ‰ as the optimal combination when assessing the effect of temperatures 14, 19, and 24 °C and the salinities 26, 32, 38 and 44 ‰ on the productivity of *Tisbe holothuriae* when fed an artificial diet and *Ulva*. High levels of mortality were exhibited by *T. holothuriae* outside of the salinity range 26 to 44 ‰ and temperature range 14 to 24 °C (Miliou,

1993). The increase in naupliar mortality of the Tasmanian *Tisbe* during the second filial generation may have been a reflection of the high culture temperature (23 °C) at which the investigation took place.

In 1991, Milou & Moraitou-Apostolopoulou conducted a comprehensive series of experiments investigating the interactive effects of both salinity and temperature on *Tisbe holothuriae* revealing:

- salinities above or below the optimum resulted in reduced productivity as a result of an extension of mean generation time (T) and net reproductive rate (R_O).
- an increase in temperature over the range 14 to 24 °C induced an increase in T and R_O.
- temperature and salinity interactions over the range 14 to 24 °C and 26 to 44 ‰ were not found to be significant.
- longevity was shortened with increasing temperature.
- longevity increased with increasing salinity to optimum, further increases resulting in a reduction of longevity
- salinity effects on longevity were more pronounced than temperature effects.
- temperature had a more important effect on generation time.

My results followed similar trends. Deviation from the optimal temperature and salinity combination (20 °C and 35 ‰) resulted in reduced productivity as a result of reduced sex ratio and net rate of reproduction. Decreasing sex ratio (CVI female to CVI male) is indicative of aging and unhealthy culture populations (Lazzaretto, 1994). Zhang & Uhlig (1993) found no clear relationship between sex ratio and population density. Mean generation times would also have been negatively affected either side of the optimum. The action of temperature and salinity were independent on all demographic parameters measured. However, the effects of either temperature or salinity can modify, and be modified by the other (Kinne, 1971).

The results obtained here from the investigation into the effect of water exchange on *Tisbe* population growth when cultures were maintained near optimal conditions (20 °C and 35 ‰) suggest that a high degree of water exchange is not critical to *Tisbe* culture health. The benefit of water exchange may have become more important with higher culture population densities. Fava & Crotti (1979) found density to affect naupliar production in *T. clodiensis* and *T. holothuriae*, the daily renewal of water diminishing the effects of crowding. A stronger inhibitory effect on naupliar production was observed with increased culture density above 400

individuals L^{-1} (Fava & Crotti, 1979). Water exchange was also observed to facilitate faster development in *T. holothuriae* by Zhang & Uhlig (1999).

The importance of biofilms as a food source for *Tisbe* may have been a confounding influence; also the higher frequency of water exchange was not directly compensated for by the addition of food algae. The use of heterotrophic diets may require more frequent water exchange to preserve water quality as a result of their tendency to promote the deterioration of water quality and an increase in bacterial blooms.

It has been suggested that a varied diet has a greater chance of meeting nutritional requirements. *Rhodomonas lens* and *Isochrysis galbana* which singly supported 5 and 9 filial generations, in combination supported over 350 germ free generations of *Tigriopus japonicus* in the last ten years (Provasoli et al., 1970). Weiss et al. (1996) found that although lipid-rich diets support the greatest productivity of the harpacticoid *Nitocra spinipes*, the presence of lipid in the diet was found not to be essential for continued reproduction and population increase. Similarly nauplii of *Tisbe holothuriae* reared on diets with dissimilar HUFA contents did not reflect the difference (Norsker & Støttrup, 1994). These findings lend support to the theory that *Tisbe* is an omnivorous opportunist well able to sustain population growth on a variety of diets. The diet chosen for use in the production of *Tisbe* as a live food for larviculture may then be largely dictated by convenience and hygiene considerations.

The decision to focus on microalgal diets in the research undertaken is rendered credible on the basis of the results obtained when microalgal diets were assessed against artificial fish foods.

Population densities equivalent to 1,280 *Tisbe* L^{-1} were achieved on a diet of *Isochrysis* over nine days at 22 °C and 35 ‰, similar to the results achieved in the temperature by salinity trial. Cultures maintained at 20 °C produced around 900 individuals L^{-1} after nine days when fed a diet of mixed diet of *Isochrysis*, *Tetraselmis* and fish crumble. The benthic diatom *Nitzschia* was found to support *Tisbe* productivity comparable with that achieved using *Tetraselmis* and *Isochrysis*.

The use of small volume static cultures exacerbated the water polluting effects of the artificial NRG4 and salmon feed diets (Appendix A4). The productivity of the cultures may have been improved by using a smaller ration and implementing a degree of water exchange.

The main trial assessing *Tisbe* performance on a variety of diets utilised a reduced ration of artificial food in combination with a flow-through system benefiting from a slightly larger culture volume and the daily introduction of fresh food and clean culture medium. The 500 mL culture units with a reservoir and screened overflow outlet may well have resulted in higher population densities than that achieved in

the static cultures as a result of the dilution of growth inhibiting compounds and maintenance of food levels.

The most productive *Tisbe* cultures in terms of total numbers and nauplii production were identified as 20 °C and 35 ‰ when fed a mixed microalgal diet comprising *Isochrysis* and *Tetraselmis* at 10^5 cells mL^{-1}. As population density increases above 2,000 individuals L^{-1}, the provision of aeration or increased water flow may prove beneficial to continued productivity at higher culture densities.

2.4.3 Larval fish feeding trials with *Tisbe*

Larval feeding trials conducted with greenback flounder and *Tisbe* demonstrated that flounder larvae:

- accepted and ingested copepod naupliar and copepodid stages,
- exhibited a strong preference for *Tisbe* nauplii at the commencement of exogenous feeding,
- exhibited a higher feeding incidence when presented with *Tisbe* in combination with rotifers and Artemia, than when presented either rotifers or *Artemia* alone,
- targeted successive life stages of *Tisbe* with increasing age,
- of all ages investigated (4 dph through 33 dph) were able to tolerate individual transfer by pipette to experimental systems followed by 17 hours without food prior to the commencement of feeding trials, exhibiting zero mortality rates,
- had recently commenced feeding were prone to loose some of their gut contents when preserved within three hours of commencing exogenous feeding,
- gut contents are easily visible and distinguishable under a dissecting microscope (40x) using a cover slip to assist gut evacuation by the larvae.

The ultimate aim of the series of experiments was to undertake a 35 day trial assessing the survival, growth and weaning success of flounder reared on four diets: a) an unfed control, b) *Tisbe*, c) the traditional rotifers and *Artemia*, and d) a traditional diet supplemented with *Tisbe*. Redirection of the focus of research to tropical species combined with unreliable supplies of *Tisbe* and flounder larvae, meant the trials were not completed. However the acceptance of all life stages of *Tisbe* by flounder of 5 to 33 dph has been demonstrated, with first feeding flounder larvae consistently demonstrating a preference for *Tisbe* nauplii over rotifers, consistent with Northern Hemisphere observations (Kuhlmann et al., 1981; Checkley, 1982; Van der Meeren, 1991).

At the onset of exogenous feeding flounder larvae expressed a preference for *Tisbe* nauplii over rotifers similar to the phenomenon reported by Kuhlmann et al. (1981) and Van der Meeren (1991) who demonstrated a preference by turbot for harpacticoid nauplii in preference to calanoid nauplii and rotifers. First feeding flounder larvae targeting *Tisbe* nauplii were also observed to exhibit a higher rate of feeding success than those presented rotifers. *Tisbe* nauplii and rotifers exhibit significantly different modes of movement and colour, in addition to size disparity. A greater number of *Tisbe* nauplii were consumed per larvae when present in isolation than when presented as a component of a mixed diet.

Feeding success of larvae is often as low as 6 to 10% at the onset of feeding (Hunter, 1981). The gut content of first feeding larvae on mixed prey is also low – the presence of different prey notably confused the larvae with a consequent increase in prey handing time and a decrease in feeding rate (Cunha & Plana, 1995).

Similarly at 8 dph, of the larvae presented with either copepods, rotifers, *Artemia* or a mixture, 100% feeding was only exhibited by those larvae presented the mixture or rotifers. Feeding percentages associated with copepods being 95%, and *Artemia* being 75%. This may be an artifact of conditioning to a diet of rotifers during the four days between trials. Cox & Pankhurst (2000) demonstrated positive selection for familiar prey, indicative of a learned feeding behaviour, adding further support to the selective feeding demonstrated by fish larvae (Checkley, 1982; Meyer, 1986).

An increase in the size of prey selected by marine fish larvae as they grow is well documented in the literature and occurs in every species studied (Dewtwyler & Houde, 1970; Hunter, 1981; Ghan & Sprules, 1993; Fernández-Diaz et al., 1994). Shaheen et al. (2001) found larger prey became more important as winter flounder fish size increased with the newly settled winter flounder targeting larger calanoid copepods.

Flounder larvae at 8 dph were observed to consume all three live foods (rotifers, *Tisbe* and instar I *Artemia*) the numbers of each consumed decreasing in the same order.

The observation of a slightly higher number of prey items from the gut contents of preserved 8 dph larvae in contrast to those processed immediately without preservation may be attributable to preservation arresting digestive processes immediately. The difference was only significant with respect to the number of *Tisbe* counted from flounder gut contents, which may be a reflection of the greater digestibility of copepods compared to rotifers and *Artemia* (Person Le Ruyet, 1989; Munilla-Moran et al., 1990; Kibria, et al., 1997).

Flounder of 15 dph consumed significantly more live food items when presented rotifers, *Artemia* and *Tisbe* as a mixture than when offered any of these food items individually. In contrast the number of rotifers and *Artemia* counted from the gut contents of 25 dph flounder were significantly greater than the number of live food retrieved when the flounder were fed either a mixed diet, or *Tisbe* in isolation. The apparent anomaly may be explained by the preconditioning of the flounder to rotifers and *Artemia* over the ten days between trials. Cox & Pankurst (2000) confirming the effects of larval familiarity with expressed live food preference.

The *Tisbe* life stages presented to the older flounder consisted mostly of later stage copepodids which are significantly larger than the rotifers and *Artemia* which, in combination with the significantly different locomotary patterns of the three live foods, and the more digestible nature of the copepod, may explain the reduced number of *Tisbe* counted.

As flounder develop from larvae through to juveniles, they naturally select progressively later developmental stages of copepods; that is nauplii through to ovigerous females. Osse (1990) observed strong selectivity of fish larvae for bigger prey up to limits set by mouth size and handling ability.

The trends from my preliminary trials conducted with flounder of various ages from different spawnings were observed in the responses of flounder sampled weekly from the same spawning reared in the same larval rearing tank.

First feeding larvae unable to consume *Artemia* exhibited a strong preference for *Tisbe* nauplii. After six days of development and exposure to a diet of rotifers, the live food of choice was rotifers, one third the number of *Tisbe* nauplii counted from the gut contents of feeding 12 dph flounder, with no *Artemia* recorded from larval gut contents. It is usual for flounder to be weaned onto *Artemia* around 10-12 dph, the larvae used in the trial having been exposed to low densities of *Artemia* for at least two days prior to the completion of the trial.

The failure of the flounder of 12 dph to ingest significant numbers of *Artemia* may be explained by the small larval size. Flounder were 0.2 mm shorter than the 8 dph larvae of the preliminary trials, with 50 µm shorter jaws corresponding to smaller mouth gapes unable to accommodate instar I *Artemia* nauplii.

Flounder of 19 dph continued to exhibited a strong preference for rotifers consuming seven and four times more rotifers than *Tisbe* copepodids and *Artemia* nauplii respectively. Rotifers continued to be the most numerous live food recorded from the gut contents of flounder aged 26 and 33 dph. Greater difficulty was experienced in distinguishing discrete food items in the gut contents of the flounder, the mastax of the rotifers being the only part of any live food items resistant to digestion.

The increase in the number of *Tisbe* counted from 33 dph flounder may be due in part to the post-metamorphic flounder being more surface orientated than younger flounder. *Tisbe* copepodids are known to be surface orientated in later stages (Støttrup & Norsker, 1997), which when combined with the greater visual acuity of the flounder and the more obvious nature of ovigerous *Tisbe*, may explain the increase in the number of larger *Tisbe* observed in the gut contents of the 33 dph flounder.

2.4.4 Summary of findings for *Tisbe*

The Tasmanian species of *Tisbe* demonstrates many characteristics similar to those documented in the literature for Northern Hemisphere counterparts and exhibits considerable potential as a live food for larviculture. In terms of the objectives identified in the introduction:

- *Tisbe* exhibits a typical harpacticoid life cycle with twelve free swimming stages ranging in size from 80 µm up to 825 µm in length with a range in width from 67 µm to 355 µm.

- Initial separation of stages based on significant dimensional differences are able to be further refined using gross morphological features including segmentation and morphology of antennules and urosome (Table 2.4.1).

- *Tisbe* exhibits significant live food potential in terms of food particle size possessing at least two nauplius stages (NI and NII) with widths less than 100 µm (Table 2.3.1).

- *Tisbe* exhibits a mean generation time in the order of 7 days at 22 °C, naupliar stages being present for three of these.

- All twelve life stages of *Tisbe* express strong negative phototactic behaviour, which is of potential value in the harvest of stages for use as live food.

- Culture populations maintained at 35 ‰ and 20 °C and fed a diet of *Isochrysis* and *Tetraselmis* at a cell density of $1\text{-}2\text{x}10^5$ cells mL^{-1} achieve densities of 6,000 *Tisbe* L^{-1}. Aeration is beneficial to maintaining populations at densities greater than 2000 *Tisbe* L^{-1} and at suboptimal combinations of temperature and salinity.

- Environmental and food conditions exert significant influences on the population as a whole. The closer to optimal the culture conditions, the greater the population density sustained and the more consistent are the numbers of nauplii present in the population.

- Larvae of the greenback flounder exhibit a strong preference for *Tisbe* nauplii at first feeding.

- Greenback flounder will ingest all life stages of *Tisbe*, the size of stages ingested increases with the age of the flounder larvae. However, no significant selection for *Tisbe* is made beyond first feeding when older flounder larvae are exposed to *Tisbe* as a novel live food.

Chapter 3
Apocyclops dengizicus

3.1 Introduction

The majority of free-living cyclopoids that inhabit saline waters are members of the families Cyclopidae, Cyclopinidae and Oithnidae (Huys & Boxshall, 1991), *Apocyclops dengizicus* being a brackish water form of the largely freshwater Cyclopidae (Valderhaug & Kewalramani, 1979).

The elementary taxonomic details of *A. dengizicus* are listed in Table 3.1.1.

Table 3.1.1: The taxonomic hierarchy for the genus *Apocyclops* (from Bowman & Abele, 1982; Huys & Boxshall, 1991).

Level	Title	
Subclass	Copepoda	Milne-Edwards, 1840
Superorder	Podoplea	Giesbrecht, 1882
Order	Cyclopoida	Burmeister, 1834
Family	Cyclopidae	Dana, 1853

Apocyclops dengizicus has been reported from the tropical and temperate regions of Asia, Africa, South America, North America and throughout Europe (Valderhaug & Kewalramani, 1979; Mirabdullayev & Stuge, 1998). It has also been recorded previously from two locations in Australia: Lake Buchanan, Queensland, (Keifer, 1967; Timms, 1987) and Paroo, New South Wales approximately 900 km southwest of Lake Buchanan (Timms, 1993).

3.1.1 Background information for *Apocyclops* species

Apocyclops attracted attention as a potential live food candidate with its presence in barramundi larval rearing ponds in the Northern Territory (Appendix B) and its apparent robustness under artificial culture conditions. *A. dengizicus* tolerates environmental fluctuations (Dexter, 1993; Timms, 1993) and exhibits "weed-like" characteristics as a frequent cohabitant of rotifer cultures at the DAC. However barramundi farmers in the Northern Territory were concerned about the potential predatory nature of the copepod in their green-water larval rearing ponds. Farmers related personal observations of cyclopoid copepods attacking live barramundi, and they attributed poor barramundi larval survival rates to copepod predation.

There is very little published information relating to the culture of *Apocyclops* species, the majority of published material relevant to *A. dengizicus* originates from the Northern Hemisphere and it relates to geographical distribution, taxonomy and value in the biological control of mosquito larvae (Timms, 1987; Mirabdullayev & Stuge, 1998; Reid & Marten, 1995). Congeneric species such as *A. royi* have been used by private hatcheries rearing grouper in Taiwan (Su et al., 1997; Liao et al.,

2001) and *A. borneoensis* exhibited potential as a replacement for *Artemia* in Malaysia (James & Al-Khars, 1984). *A. panamensis*, a common inhabitant of brackish and coastal lagoons and ponds around the Gulf of Mexico and Caribbean, is of interest as a predator of mosquito larvae (Reid & Marten, 1995).

Populations of *Apocyclops* have been cultured by Valderhaug & Kewalramani (1979) over the range of temperatures from 20 to 28 °C and in 200 mL non-aerated cultures without food for up to 120 days at 20 to 25 °C over the salinity range 0.5 to 68 ‰ by Dexter (1993). Successful reproduction for at least one generation was observed between 0.5 and 68 ‰, with inoculated individuals surviving, but not reproducing, at salinities of up to 79 ‰ (Dexter, 1993).

Little information has been reported concerning the diet of *Apocyclops dengizicus*, however this cyclopoid has been observed to exhibit similar characteristics to *Tisbe*, appearing to be an omnivorous opportunist. As the majority of cyclopoids have no trace of any true filtration mechanism in the oral region, Davis (1984) deduced they must therefore obtain their food by grasping individual particles or by attacking larger prey raptorially, an observation already made by Allan (1976). Valderhaug & Kewalramani (1979) successfully reared *A. dengizicus* on *Dunaliella primolecta* with Dexter (1993) indicating *Brachionus plicatilis* to be a suitable food. Shigur (1989) reported *A. dengizicus* responded well in pond culture to fertilisation using organic slurry.

Work undertaken with *Apocyclops royi* indicates *Tetraselmis chuii* densities greater than 10^5 cells mL^{-1} corresponded to excess feeding conditions for the cyclopoid (Chang & Lei, 1993). Chang & Lei also report personal observations of *A. royi* preying on *Artemia* nauplii greater than 1 mm in length, the natural diet of *A. royi* including the eggs and gravid segments of the cestode *Hymenolepis gracilis*. *A. panamensis* adults have been observed to shift from seston to carnivorous feeding depending on the availability of food (Guiral et al., 1994; Pagano et al., 1999), with populations of *A. borneoensis* successfully cultured on baker's yeast at 20 mg L^{-1} day^{-1} (James & Al-Khars, 1984) lending support to the idea of omnivorous feeding for *Apocyclops*. The possible inclusion of fish larvae in the diet of *A. dengizicus* may be demonstrated by another relative, *Mesocyclops*, which has been reported to attack larvae of striped bass (Cooper, 1996).

Dr Janet W. Reid, Research Associate in the Department of Zoology at the National Museum of Natural History, Smithsonian Institution, Washington, USA, identified the Northern Territory isolate as *Apocyclops dengizicus* (Lepeschkin, 1900). Voucher specimens comprising 10 male plus 10 ovigerous females have been deposited with the Museum and Art Gallery of the Northern Territory under the catalogue number NTM Cr012710.

Throughout the remainder of the thesis, unless stated otherwise, the name *Apocyclops* indicates reference specifically to the Northern Territory isolate of *A. dengizicus*.

3.1.2 Objectives

The aim of the chapter is to document the culture characteristics of a tropical Australian *Apocyclops* species and to assess its suitability as an alternative live food for marine finfish larvae using barramundi as a test species. Three distinct areas were investigated: (a) life cycle, (b) copepod culture and (c) assessment of copepod fish larvae interactions.

The objectives of the work undertaken were to:

A. Document the number and sizes of each of the stages in the life cycle of *Apocyclops*.

B. Identify the gross morphological features of each stage so that it is possible to differentiate developmental stages during culture.

C. Determine the mean generation time and duration of each of the life stages of *Apocyclops*.

D. Identify the influence of salinity, temperature, aeration and diet on the growth and development of *Apocyclops*.

E. Assess the effects of salinity, temperature, aeration and diet on population density and demographic variables such as population structure, net rate of reproduction and sex ratio in culture.

F. Investigate the diet of various life cycle stages of *Apocyclops*, in particular its potential for piscivory (i.e. predation on larval barramundi).

This study represents the first documentation of the life cycle of *Apocyclops* cultured under artificial conditions in Australia, and addresses the issue of copepod predation on larvae of the local barramundi. The accompanying work reported in Appendix B documents information on the faunal composition of zooplankton populations developing under semi-extensive green-water aquaculture conditions in the Darwin region of the Northern Territory.

3.2 Materials and methods

Common methods

Throughout the chapter all water quality parameter measurements were taken using the same equipment. Similarly, unless stated otherwise, the algal culture conditions detailed below were used.

Water Quality Assessment

Temperature (°C) and dissolved oxygen levels (DO, $mgO_2\ L^{-1}$ or %) were measured using a WTW Oxi320 oxygen meter calibrated daily and accurate to within 0.2 $mgO_2\ L^{-1}$. The WTW Oxi320 temperature probe was calibrated according to manufacturer's instruction immediately after purchase. Two subsequent calibration checks confirmed accuracy after three years.

Salinity, recorded in parts per thousand (‰), was measured using an ATAGO S-10 hand held refractometer accurate to 0.5‰ calibrated prior to use with temperature acclimated freshwater.

pH levels (± 0.01 pH units) were measured using a Hanna Instruments pH meter (HI 8424) calibrated daily.

Nitrate and nitrite levels were determined using the corresponding HACH colourimetric test kits for salt water systems. The levels of nitrite (mg $NO_2\ L^{-1}$) and unionised ammonia (mg $NH_3N\ L^{-1}$) could be detected if present at levels above and below those concentrations considered to be deleterious to larval fish performance.

Algae Culture & Maintenance

All species of algae were batch cultured in the Darwin Aquaculture Centre algal laboratory at 26°C, 30‰, under a 14L:10D photoperiod regime between 17,000 and 20,000 lux (330-590 $\mu mols^{-1}m^{-2}$) and fertilised with f_2 medium (Guillard & Ryther, 1962). Seawater for algal cultures was successively filtered through sand and then through 5, 1 and 0.2 µm cartridge filters followed by exposure to ultra-violet light.

Stock cultures were maintained in 500 mL flasks and scaled up through 20 L polycarbonate carboys to 300 L plastic bags. Each 300 L culture was fed to rotifer and copepod cultures for a period of 2 to 3 weeks, during which time the bags were refilled with filtered seawater and refertilised as required.

Four species of single celled microalgae were used in subsequent trials with *Apocyclops*:

a. *Rhodomonas* sp. (Northern Territory University collection no. NT15) isolated from Darwin Harbour exhibiting a mean cell size of 9.2 x 5.6 µm is referred to as *Rhodomonas* in the remainder of the chapter.

b. *Tetraselmis* sp. (CSIRO collection no. TEQL01) with a mean cell size of 13.4 x 8.4 µm is referred to as *Tetraselmis* in the remainder of the chapter.

c. *Isochrysis* sp. (Tahitian isolate CSIRO collection no. CS177) with a mean cell diameter of 6 µm is referred to as *Isochrysis* for the remainder of the chapter.

d. *Heterocapsa neie* (CSIRO collection no. CS36) exhibiting a mean cell size of 19.6 x 12 µm is referred to as *Heterocapsa* in the remainder of the chapter.

3.2.1 Life cycle and demographics

Knowledge of the life cycle of an organism and the characteristics of each life stage in the cycle are essential to understanding the requirements of each stage (Trujillo-Oritz, 1986) and the successful culture of the species. *Apocyclops* has not specifically been used in the Australian aquaculture industry and is therefore an unknown entity. However the presence of the copepod amongst the zooplankton in green-water barramundi ponds (Appendix B) and in rotifer cultures suggests that *Apocyclops* may be suited to intensive culture. The objectives of the following investigations were to observe the life cycle of *Apocyclops* and identify key characteristics of importance to aquaculture technicians in the culture and use of *Apocyclops* as a live food for marine finfish larviculture.

Apocyclops collection and culture

Adult *Apocyclops* were obtained from rotifer cultures originating from green water barramundi rearing ponds at the commercial farm, Barramundi Farms NT located west of Darwin city, Northern Territory, Australia. The copepods were separated from the rotifers by passing the mixed culture over a 150 µm mesh screen. The copepodids retained on the screen were rinsed in seawater of 30 ‰ for a period of three hours in the 'plankton washer' (Section 3.2.1.1) fitted with a 150 µm mesh to remove any rotifers.

The *Apocyclops* copepodids isolated thus were transferred to a 100 L culture unit comprising a 100 L Nally® bin prepared with 60 L of 1 µm filtered seawater at 30 ‰ with *Isochrysis* and *Tetraselmis* at a final density of $1x10^5$ cells mL^{-1} and 0.01g of fish mince. The culture was maintained outside the laboratory under ambient light conditions corresponding to a photoperiod of 13 L:11D and a temperature range of 29 to 32 °C.

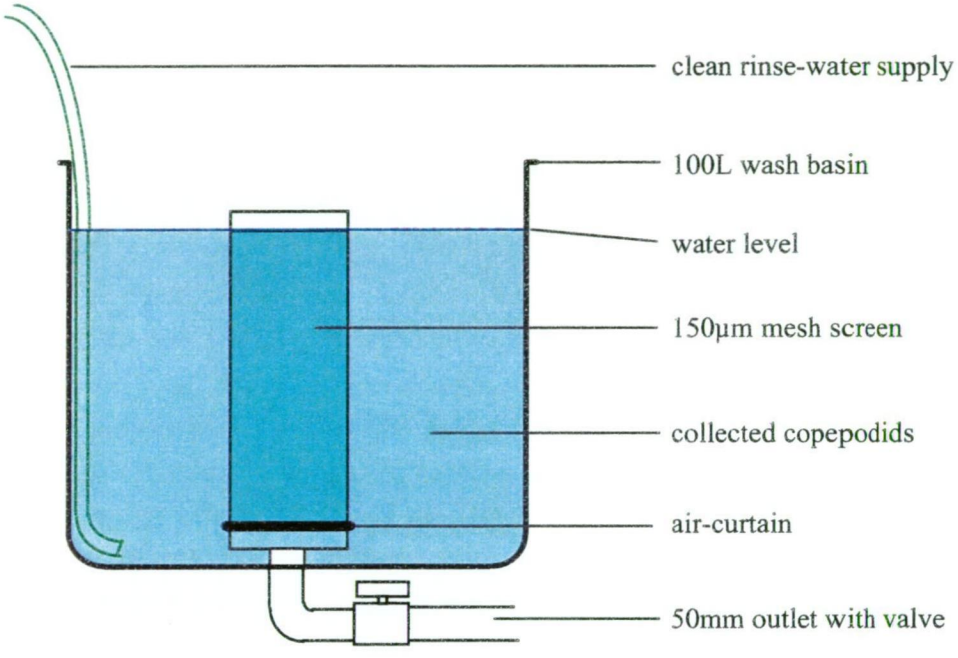


Figure 3.2.1: Diagram of the 100 L 'plankton washer'. Clean 1 µm filtered, ultra-violet sterilised rinse seawater was passed through a 100 L wash basin containing the collected copepods. The excess water passed through a central standpipe (located within the screen) which dictated the water level during rinsing. An air-curtain created by a perforated 4 mm airline wrapped around the bottom of the central screen which supported the appropriate size mesh improved water flow-through the screen. Once the copepodids have been sufficiently rinsed, the outlet valve was closed and the water supply, central screen and standpipe removed. Copepodids were then either scooped out, or the outlet valve opened and the copepodids collected over a net in a basin.

3.2.1.1 Life cycle

Ovigerous *Apocyclops* were isolated from the main culture and used to establish a stock culture maintained under the same conditions. The original females were removed from the stock culture after four days by passing the contents over a 150 µm mesh screen. The early stage copepodids and nauplii which passed through the screen were returned to the culture for a further five days, after which eighteen ovigerous *Apocyclops* were selectively siphoned from the stock culture using a 4 mm diameter siphon hose. These females were transferred to individual culture units containing 20 mL of fresh culture media as for *Tisbe* (Section 2.2.1.1).

The *Apocyclops* females used were of known age and history in an attempt to reduce variation in the data obtained from the developing progeny. The *Apocyclops* females and resultant nauplii were treated in the same manner as the *Tisbe* females and their progeny (Section 2.2.1.1).

3.2.1.2 Demographics and productivity

Fifteen ovigerous *Apocyclops* females were isolated and introduced to individual culture units and monitored daily for the extrusion and hatching of egg sacs. Upon hatching of each egg sac, females were individually transferred by Pasteur pipette to fresh culture media in separate individual culture units. The hatched nauplii were retained in the initial culture vessel and assigned a label comprising the numeric female identifier and a serial alphabetic egg sac identifier as was done for *Tisbe*. All *Apocyclops* cultures were monitored as described previously for *Tisbe* (Section 2.2.1.2) over three weeks allowing the development of the F_1 progeny and the death of the F_0 females.

Statistical Analyses

Statistical analyses as described for *Tisbe* in Section 2.2.1 were employed to ascertain the value of life stage size as a diagnostic character.

3.2.2 Culture of *Apocyclops*

Apocyclops exhibits many similar physical traits to *Tisbe*, such as robustness and tolerance of small culture volumes, enabling use of techniques similar to those outlined for that copepod in Section 2.2.2.

The responses of *Apocyclops* to rapid fluctuations in salinity, and the influence culture salinity over the range from 10 ‰ to 50 ‰ were assessed in static (non-aerated) culture systems. The influence of salinity on *Apocyclops* populations was also assessed in flow-through systems over the salinity range 15 ‰ to 45 ‰.

To obtain the maximum amount of information possible within the shortest time frame, a trial was run to collect information on a number of factors concurrently.

The influences of temperature, diet, aeration and handling on *Apocyclops* productivity were unknown. The robustness of some culture populations to handling was assessed by the removal and replacement of a portion of the population at intervals, the controls not being exposed to additional handling. Three diets comprising two algal and an artificial diet were investigated in a view to eliminating algae from future trials. Temperature fluctuations over the range likely to be encountered in the natural environment (i.e. from 23 to 36 °C) were also investigated. And in view of the positive effect of aeration on *Tisbe* productivity, the influence of aeration on *Apocyclops* cultures was also addressed.

The influence of temperature over the range 23 °C to 35 °C and diet composition on *Apocyclops* culture populations were reassessed separately. Details of the experiments are provided below.

3.2.2.1 The effect of salinity

Casual observation of *Apocyclops* stock cultures revealed that healthy copepod populations persisted for several weeks over a wide range of salinities when the cultures experienced gradual changes in salinity.

The following trials were completed to assess the tolerance of *Apocyclops* females to rapid changes in salinity, and the influence of culture salinity on *Apocyclops* populations when maintained in non-aerated and flow-through culture systems.

Trial 1 – Tolerance of Apocyclops to rapid salinity change

The objective of the trial was to assess the tolerance of ovigerous *Apocyclops* females to sharp salinity changes (i.e. increments up to 30 ‰), which could possibly be experienced as a result of inoculation of future trials. Rapid changes in salinity may also be experienced as a result of larval fish being reared at different salinities to that at which the *Apocyclops* live food cultures were being maintained to achieve optimal live food numbers.

Ninety ovigerous *Apocyclops* were selected from a stock culture maintained at 30 ‰ on a mixed algal species diet comprising *Tetraselmis* and *Isochrysis,* held at ambient temperature (30.6 °C ±0.12 °C, range 24.3 °C to 33.1 °C). The females were subsequently rinsed by pipetting each through a series of droplets of 0.45 µm filtered seawater at 30 ‰. The viability and longevity of females were unaffected by such treatment.

Duplicate cylindrical culture units were filled with 20 mL of media made up at each of the nine treatment salinities: 10 ‰, 15 ‰, 20 ‰, 25 ‰, 30 ‰, 35 ‰, 40 ‰, 45 ‰ and 50 ‰. The range of salinities was achieved through either the addition of deionised water or artificial Aquasonic® Ocean Nature marine salts to 0.45 µm filtered natural seawater originally at 30 ‰ containing a light concentration of *Tetraselmis* and *Isochrysis* (~10^4 cells mL^{-1}).

Each culture unit was inoculated with 5 ovigerous *Apocyclops* on day zero (D0). Each culture was subsequently monitored on days 1, 3, 5 and 7 for the presence of egg sacs, at which time 0.4 mL of both *Tetraselmis* and *Isochrysis* were added to each culture.

Trial 2 – The effect of salinity in non-aerated Apocyclops cultures

Apocyclops has been reported to tolerate salinities over the wide range of 4 ‰ to 128 ‰ (Timms, 1987), however reproduction was only evident over the range of 0.5 ‰ to 68 ‰ (Dexter, 1993). The objective of the trial was to identify a suitable culture salinity from within the range from 10 to 50 ‰. The range selected encompassed the most common salinity range for mariculture (20 to 38 ‰) extending beyond these to ensure that the salinity most suited to dense *Apocyclops* culture populations was included in the salinities examined.

Ten 100 L water baths adjusted to 32 ±0.2 °C were set up as described in Section 2.2.3.1 with water bath temperatures monitored daily and adjusted where necessary.

One week prior to the commencement of the experiment a stock culture was inoculated with *Apocyclops* collected from Barramundi Farms NT. Copepodids greater than 150 µm collected in trawls from ponds at the farm were thoroughly rinsed using the 'plankton washer' (Section 2.3.2) prior to their transfer to a fresh, lightly aerated 80 L culture at 30 ‰ fed a mixture of *Tetraselmis* and *Isochrysis*. Two days later the stock culture was screened over a 150 µm mesh screen to remove the inoculum copepodids leaving behind only nauplii developing from eggs sacs produced by females present in the inoculum. After five days in culture, the population was again screened over a 150 µm mesh to isolate ovigerous *Apocyclops* to be used as the inoculum for the salinity trial.

Culture media at nine different salinities (10, 15, 20, 25, 30, 35, 40, 45 and 50 ‰) were prepared with *Tetraselmis* and *Isochrysis* included at a cell ratio of 0.6:1 resulting in a final density of 1.6×10^5 cells mL^{-1}. The total volumes and salinities were adjusted using a combination of 0.1 µm filtered natural seawater, Aquasonic Ocean Nature® artificial sea salt, and acclimated tap water. The pH, nitrite and unionised ammonium levels of the fresh media were recorded prior to distribution of media to the 500 mL culture units.

Time constraints precluded all ten replicate cultures being inoculated simultaneously, resulting in the introduction of a time block to the experimental design. Five replicate cultures at each salinity were inoculated on day 1 and the remaining five replicate cultures inoculated on day 2 of the trial. On each of the days one and two of the trial, 230 ovigerous *Apocyclops* were selected from the stock culture and individually pipetted through three droplets of clean seawater at 30 ‰ to ensure rotifer-free status of the inoculum. Five ovigerous *Apocyclops*

were randomly distributed to each of the ten replicate units containing 20 mL of fresh saline water at 10, 15, 20, 25, 30, 35, 40, 45 and 50 ‰.

The ovigerous *Apocyclops* in the 20 mL volumes were checked for retention of egg sacs prior to being introduced to replicate 500 mL culture units of the corresponding salinity. One replicate culture unit from each treatment salinity was placed in each of the ten water baths maintained at 32 °C.

Every third day post-inoculation, each culture unit received an 80% culture medium exchange using freshly prepared, temperature acclimated culture media. The 400 mL of spent culture medium were siphoned off through a 44 µm mesh screen, and the salinity and pH levels of the spent media were recorded. Prior to each culture receiving 400 mL of fresh media, a 6 mL subsample of the remaining 100 mL of media was taken and the number of early stage nauplii, late stage nauplii, copepodids and ovigerous females recorded.

The trial was terminated after three weeks, with the final temperature, salinity and pH levels recorded for each culture unit. Unionised ammonia and nitrite levels were recorded for six replicates from each treatment salinity as previous experience indicated that nitrogen levels within each treatment salinity were similar. The contents of each culture unit were reduced to 50 mL by siphoning off the media through a 44 µm mesh screen. The remaining 50 mL of culture containing *Apocyclops* individuals was preserved by the addition of 12 mL of a 1:1 mixture of formalin and glycerol. The number of ovigerous (ES), female (non-ovigerous CVI), male (CVI), immature copepodid (CI-CV), late naupliar (NIV-NVI) and early naupliar (NI-NIII) *Apocyclops* stages present in each sample were counted using an Olympus SZ40 dissecting microscope.

To obtain more information on population health and the reproductive capabilities of the *Apocyclops* cultures maintained at the different salinities, sex ratios (female CVI: male CVI) and indicative net rates of reproduction (R_o) were calculated from data collected on days nine and twenty-one. Only samples containing representatives of both sexes were used when assessing the influence of salinity on sex ratio. The calculated values were unable to be transformed to meet the assumptions of ANOVA and were analysed by Kruskal-Wallis *k*-sample test.

Trial 3 – The effect of salinity in flow-through Apocyclops cultures

The following trial was designed to take into consideration the potentially confounding influences of 1) female history masking treatment effects, and 2) different salinities on algal quality. *Apocyclops* maintained at the treatment salinities for a period of one week prior to inoculation of the experiment were used to reduce the influence of female history. The trial was run in flow-through culture systems to reduce any confounding effects as a result of deteriorating algal quality

as a result of salinity. The objective of the trial was to identify the salinity most favourable to *Apocyclops* population growth.

Nine days prior to the inoculation of the trial, a population of mixed life stages of *Apocyclops* was obtained from an existing culture and evenly distributed between five 10 L cultures at salinities of 15 ‰, 20 ‰, 25 ‰, 30 ‰ and 35 ‰ containing *Tetraselmis* at $6x10^4$ cells mL^{-1} and *Isochrysis* at $1x10^5$ cells mL^{-1}. After one week the five cultures were screened to separate the nauplii and the copepodids. For each of the five salinities 160 late stage copepodids were inoculated into 2 L of culture media at the corresponding treatment salinity for 2 days during which time ovigerous females developed.

The culture system consisted of 500 mL culture units fitted with a 44 µm mesh screened overflow, each with a 1 L reservoir as used in the *Tisbe* diet trial (Section 2.2.2.5 Figure 2.2.3). Reservoirs comprised a 1 L measuring jug supplied with air via a 4 mm airline ending in a Pasteur pipette. The contents of the reservoir were gravity fed into individual culture units via a 4 mm weighted line terminating in a flow regulator resulting in fresh culture media delivery at a rate of 300 mL hr^{-1}.

Culture media were prepared at the five treatment salinities (i.e. 15 ‰, 20 ‰, 25 ‰, 30 ‰ and 35 ‰) by diluting 0.1 µm filtered seawater of 35 ‰ with the appropriate volume of deionised water. The final culture media at each salinity contained *Tetraselmis* at $6x10^4$ cells mL^{-1} and *Isochrysis* at $1x10^5$ cells mL^{-1}. Four replicate culture units were filled with 500 mL of media for each of the five salinity treatments.

On day zero (D0) of the trial, forty ovigerous *Apocyclops* were individually pipetted from each of the 2 L cultures maintained at 15 ‰, 20 ‰, 25 ‰, 30 ‰ and 35 ‰. Ten females from each of the five salinities were subsequently inoculated into the four replicate culture units for each corresponding treatment salinity (i.e. ten females at each of five salinities with four replicates).

Apocyclops cultures were maintained at 29 ±0.1 °C in a constant temperature room under a 13 L:11D regime mimicking the ambient photoperiod. Culture temperature, salinity, pH and dissolved oxygen levels were monitored daily with light levels recorded at the commencement and termination of the trial. A 200% volume water exchange was conducted daily from a 1 L reservoir filled with fresh medium of the corresponding salinity and fresh *Tetraselmis* at $6x10^4$ cells mL^{-1} and *Isochrysis* at $1x10^5$ cells mL^{-1}.

Four days post-inoculation (D4), the *Apocyclops* females used as the inoculum were removed to alleviate any reduced productivity due to cannibalism, and to enable a more accurate estimation of progeny survival rates and net rate of reproduction.

Every third day (i.e., D3, D6 and D9) a 20 mL sample was collected from all *Apocyclops* culture populations and the number of naupliar, copepodid and ovigerous stages were counted to assess population composition and health. The sample was replaced after examination under a stereo-dissecting microscope.

The trial was terminated after nine days (on D9). The environmental parameters were recorded for all cultures prior to their contents being screened over a 44 µm mesh screen. The *Apocyclops* life stages retained were preserved in a 1:1:8 mixture of formalin, glycerol and culture medium with the number of individuals in each life stage group determined as detailed in 3.2.2.1.

3.2.2.2 The effects of temperature, diet, aeration and handling

The aim of the following trial was to assess the influence of various environmental and maintenance factors on the productivity of 500 mL *Apocyclops* cultures. Factors assessed were temperature (20, 23, 26, 29, 32 and 35 °C), diet (*Isochrysis*, fish crumble and *Tetraselmis*), aeration (with and without) and handling (additional handling and minimal handling).

The design of the trial reflected the assumption that the impact of handling would be minimal compared to the effects of temperature, aeration and diet on *Apocyclops* culture population densities. Spatial and temporal constraints resulted in a nested factorial experimental design with only one replicate for each of the seventy-two temperature, diet, aeration and handling combinations (6 temperatures x 3 diets x 2 aeration x 2 handling = 72 treatment combinations).

Twelve quasi-replicate cultures in total were allocated to each of the six treatment temperatures. At each temperature, diet treatments were represented in quadruplicate. Within each diet, aeration treatments were present in duplicate. For each temperature-diet-aeration combination, one replicate was monitored regularly, and the other was left untouched to gauge the influence of handling on culture productivity. Table 3.2.1 outlines the allocation of treatment factors.

The trial was conducted in a constant temperature room maintained at 19 ±1 °C. Twenty 100 L water baths were set up as previously described in section 2.2.2a containing 60 L of water and heated by Jäger 250 Watt aquarium heaters calibrated to within ±0.2 °C of the treatment temperature. The diet replicates within each temperature treatment were distributed between the four water baths at each temperature.

Table 3.2.1: Nesting of treatment factors assessing the influence of temperature, diet, aeration and handling as illustrated by the distribution of treatments within each temperature (20, 23, 26,29, 32 and 35 °C). 20 °C is used as an example where '+' indicates aeration, '-' no aeration; '*a*' indicates additional handling applied, and 'b' minimal handling.

Temperature	Diet	Aeration	Handling
20	*Isochrysis*	+	*a*
20	*Isochrysis*	+	*b*
20	*Isochrysis*	-	*a*
20	*Isochrysis*	-	*b*
20	Fish crumble	+	*a*
20	Fish crumble	+	*b*
20	Fish crumble	-	*a*
20	Fish crumble	-	*b*
20	*Tetraselmis*	+	*a*
20	*Tetraselmis*	+	*b*
20	*Tetraselmis*	-	*a*
20	*Tetraselmis*	-	*b*

Each replicate culture unit comprised a cylindrical plastic container with a fitted lid to reduce confounding effects due to evaporative losses. Aeration was achieved by inserting a 4 mm airline through a hole drilled into the lid of each culture unit. Sealing the end of each airline and placing thirty-two holes in the final 2 cm of the tubing with a fine hypodermic needle achieved the delivery of fine bubbles. Consistency of aeration between culture units was achieved by inserting a 2 mL hr^{-1} flow regulator into the airline immediately prior to the line passing through the lid of each unit. Culture units were supported by polystyrene skirts to ensure they remained in an upright position when floated in the water baths (Figure 3.2.2).

On the day of inoculation (D0), *Apocyclops* were collected from Barramundi Farms NT using a 44 µm-mesh hand net. One thousand five hundred ovigerous *Apocyclops* were isolated under a dissecting microscope. The females were rinsed in fresh 0.1 µm filtered seawater to eliminate contaminant rotifers, prior to being transferred into fresh 0.1 µm filtered seawater at 30 ‰ using a Pasteur pipette. Twenty clean ovigerous *Apocyclops* were randomly distributed into each of 72 vessels containing 10 mL of fresh 0.1 µm filtered seawater at 30 ‰.

The three diets were prepared in 1 µm filtered seawater at 30 ‰. Approximately 0.02 g of 1 mm barramundi fish crumble (Gibsons, now Skretting Australia,

Hobart, Tasmania) was added to each culture unit designated as being fed 'fish crumble' and filled with seawater. Culture units designated '*Isochrysis*' and '*Tetraselmis*' treatments received the corresponding algae at 1-2 x10^4 cells mL^{-1}.

The pH, dissolved oxygen concentrations (DO%), ammonia (mg unionised ammonia L^{-1}) and nitrite (mg NO_2 L^{-1}) levels of each diet culture medium was recorded prior to their inoculation at random with rinsed ovigerous *Apocyclops* and placement in the appropriate water bath. Light levels were minimal (~30 lux) for the duration of the trial due to the constraints imparted by a concurrent oyster conditioning trial.

On days 4, 8 and 12 post-inoculation, all cultures received an 80% culture medium exchange with temperature-acclimated culture media of the appropriate composition as described in the previous trial. The pH, DO, ammonia and nitrite levels of the exchange media were monitored prior to their distribution.

The handling treatment was conducted at the time of the water exchange. The 100 mL of culture medium containing the *Apocyclops* was swirled and a 20 mL sample poured off. The number of copepodids, nauplii and ovigerous *Apocyclops* present in the sample were counted under a stereo-dissecting microscope and the sample returned to the culture unit from which it was taken.

The trial was terminated after 14 days. The salinity, pH, DO, ammonia and nitrite levels were determined for five of the ten replicate culture units. The entire 500 mL of culture was screened through a 44 µm-mesh screen and the collected *Apocyclops* life stages thus collected were transferred to labelled 70 mL specimen bottles with 20 mL of culture medium and made up to 25 mL with a 1:1 mixture of formalin and glycerol preservative. The number of individuals of each life stage group was determined as detailed in 3.2.2.1.

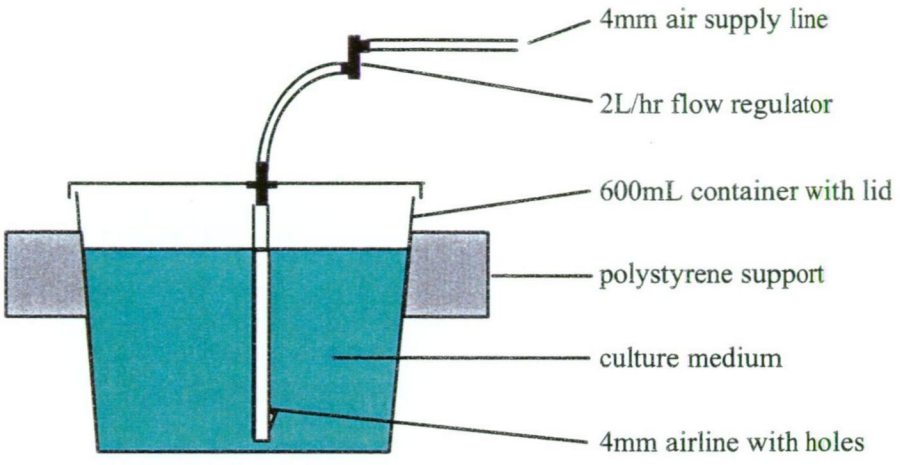


Figure 3.2.2: A diagram of the 500 mL mass culture unit.

3.2.2.3 The effect of temperature

The productivity of *Apocyclops* cultures was investigated over the temperature range 23 to 35 °C. Both mass cultures (500 mL) and individual cultures (20 mL) were included in the design to obtain confirmation of results obtained in the preceding trial and more detailed demographic information.

Fifteen 100 L water baths were set up and maintained at 22 ±2 °C in a room with a regulated temperature. Three water baths were calibrated to each of five treatment temperatures (i.e. 23 °C, 26 °C, 29 °C, 32 °C and 35 °C) over a period of one week. Temperatures were monitored twice daily throughout the trial, and adjusted where necessary.

Apocyclops were collected from an established prawn pond on Barramundi Farms NT using a 44 µm mesh throw net. The concentrated copepods were held overnight in an 80 L bin with moderate aeration. The following morning 330 ovigerous *Apocyclops* were isolated, and rinsed twice in 1 µm filtered seawater adjusted to 30 ‰, and ten ovigerous *Apocyclops* were randomly distributed to each of thirty-three 70 mL vessels containing 10 mL of seawater.

Eighteen 20 mL individual cultures units (ICU), each inoculated with a single ovigerous *Apocyclops*, were monitored daily for egg sac production and time of hatching. Each ICU consisted of a cylindrical 70 mL plastic container with a screw top lid. Three ICUs were floated in the nine water baths maintained at 23 °C, 29 °C and 35 °C. Replicate ICUs were not included at 26 °C and 32 °C due to time constraints. Once nauplii had finished hatching from a particular egg sac, the female was transferred to a separate ICU with 20 mL of fresh medium leaving her

progeny in the original 20 mL ICU which was labelled accordingly as per the demographics study in Section 2.2.1.2.

Thirty 500 mL mass culture units (MCU) based on those depicted in Figure 3.2.2 were inoculated with 10 ovigerous *Apocyclops* to obtain overall culture productivity information. The six MCU were randomly distributed between three water baths at each temperature. Changes in population density and composition were recorded after three weeks.

Culture media for both ICU and MCU was made up at 30 ‰ with *Tetraselmis* and *Isochrysis* at cell densities of $6.0x10^4$ and $1.0x10^5$ cells mL^{-1} respectively. The dissolved oxygen concentration, nitrite and ammonia levels of the fresh media were recorded prior to the distribution of 500 mL volumes to each of thirty culture units.

An 80% culture medium exchange was conducted on the fourth (D4), eleventh (D11) and eighteenth (D18) days of the trial using freshly prepared, temperature-acclimated culture media. Four hundred millilitres of spent culture medium were siphoned off through a 44 µm mesh screen. The salinity, pH and DO levels of the spent media were recorded, and the number of ovigerous females, copepodids and nauplii counted under an Olympus stereo-dissecting microscope prior to the replenishment of culture media. Ammonia and nitrite levels were also monitored in spent media obtained from two replicates maintained at each treatment temperature.

The trial was terminated after twenty-one days. Final salinity, pH and dissolved oxygen levels were recorded for each MCU. Ammonia and nitrite levels were recorded for six randomly selected MCU replicates at each treatment temperature. Each MCU was screened down to a volume of 50 mL and the remaining 50 mL of culture containing all copepodid stage was preserved in a 1:1:8 mixture of formalin, glycerol and culture medium with the number of individuals in each life stage group counted as detailed in 3.2.2.1.

3.2.2.4 The effect of diet

Results from the multi-factorial trial assessing fish crumble as an alternative to *Isochrysis* or *Tetraselmis* (Section 3.2.2.2) revealed *Apocyclops* populations maintained on microalgal diets were far more productive than those maintained on dried fish food. Subsequent diet trials did not pursue the use of non-algae based diets as a consequence of 1) the greater ease with which algal rations may be manipulated, 2) the beneficial effects of algae on water quality in comparison to the potential for putrefaction of some heterotrophic and processed foodstuffs, and 3) the consideration that the majority of hatcheries possess algal culture facilities. The following trials focus on the effects of algal species and cell density on *Apocyclops* culture density.

Apocyclops stock culture maintenance

A moderately aerated 80 L stock culture of *Apocyclops* was maintained at 29 °C and 25 ‰ on a mixed diet of *Heterocapsa*, *Isochrysis*, *Rhodomonas* and *Tetraselmis*. Algae were fed in a 1:1:1:1 cell ratio to maintain a final density of approximately $2x10^5$ cells mL^{-1}.

On a weekly basis, the aeration was removed and the culture was allowed to settle. An incandescent light was placed near the surface of the culture to draw the cyclopoids away from the detritus on the bottom. The majority of the detrital matter was siphoned out of the culture vessel with 50% of the culture volume. The culture volume was then replenished with a mixture of *Heterocapsa*, *Isochrysis*, *Rhodomonas* and *Tetraselmis* at 25 ‰.

Trial 1 – The influence of diet composition on Apocyclops populations

The objective of the trial was to assess the effect on *Apocyclops* population density of four algal species presented individually or as a mixed group.

Five gently aerated 10 L conditioning cultures were set up at a constant temperature of 29 °C, each in a 20 L cylindrical white plastic vessel fitted with a weighted 4 mm airline and a 2 mL $hour^{-1}$ flow regulator. Five different culture media were prepared - four mono-specific algal diets of *Heterocapsa*, *Tetraselmis*, *Isochrysis* and *Rhodomonas*, and one mixed algal species diet comprising a mixture of all four algae in a 1:1:1:1 ratio. All algal culture media were made up to a final algal density of $2x10^5$ cells mL^{-1}. Each 10 L culture was inoculated with 100 *Apocyclops* adults taken from the stock culture and left to condition for 3 days after which time the cultures were passed through a 150 µm mesh screen, the copepodids retained on the screen discarded. The nauplii and early stage copepodids thus retained were returned to fresh culture media. The ovigerous *Apocyclops* appearing two to three days later were used for the experiment.

Three litres of culture media were made up at a salinity of 25 ‰ according to Table 3.2.2 and left to equilibrate to 29 °C prior to their distribution between the six replicate 500 mL culture units. Each culture unit was fitted with a 63 µm-mesh overflow and an aerated 1 L reservoir fitted with a gravity feed line terminating in a 2 mL hr^{-1} dripper regulating algal discharge rates to 300 mL hr^{-1} as described in Section 3.2.2.3. Culture units were randomly distributed between two blocks within the constant temperature room maintained at 29 ±0.1 °C.

Seventy-five ovigerous *Apocyclops* carrying their first egg sacs were isolated from each of the five 10 L conditioning cultures and rinsed in 0.1 µm filtered seawater of 25 ‰ salinity. Sixty of the seventy ovigerous *Apocyclops* removed from each of the four conditioning cultures were used to inoculate the six replicate culture units for the corresponding algal treatment diet. For example, ten *Apocyclops* removed from *Heterocapsa*-fed conditioning cultures were inoculated into each of the six

replicate culture units corresponding to the *Heterocapsa* diet treatment. The ten ovigerous *Apocyclops* remaining from each of the 10 L conditioning cultures were randomly distributed between the six unfed replicate culture units, giving a total of ten in each.

Table 3.2.2: Treatment algal cell densities used in diet composition trials conducted with *Apocyclops*.

Diet	*Isochrysis* cells mL^{-1}	*Heterocapsa* cells mL^{-1}	*Rhodomonas* cells mL^{-1}	*Tetraselmis* cells mL^{-1}
Isochrysis	$1.9x10^5$	-	-	-
Heterocapsa	-	$1.9x10^5$	-	-
Rhodomonas	-	-	$1.9x10^5$	-
Tetraselmis	-	-	-	$1.9x10^5$
Mixture	$4.75x10^4$	$4.75x10^4$	$4.75x10^4$	$4.75x10^4$
Unfed	0	0	0	0

The contents of each reservoir were replenished on a daily basis with the corresponding fresh algal diet media. Prior to any media exchange, temperature, salinity, pH and dissolved oxygen levels of twelve representative cultures were monitored. Only two pots from each treatment, one from each block, were monitored as opposed to all thirty-six in an attempt to manage time constraints. Previous trials during which the environmental parameters of all pots were measured exhibited no significant difference within each algal diet treatment. Light levels at the culture surface were recorded at the commencement and termination of the trial.

Every third day (i.e. D3, D6 and D9) of the trial, all culture unit populations were monitored. A 20 mL sample was removed from each culture using a wide bore syringe (Ø 3 mm). Copepod stages were examined under a stereo-dissecting microscope to assess population composition and vigor. The sample was replaced.

Inoculum females were removed on D4 prior to the maturation of nauplii which hatched from eggs sacs on D1 of the trial to minimise the effects of cannibalism and enable the survival rates of progeny to be quantified.

The trial was terminated after nine days (on D9). Environmental parameters were recorded for all cultures prior to the addition of formalin and glycerol. The preserved *Apocyclops* were collected over a 44 µm mesh screen and retained in 20 mL of a 1:1:8 mixture of formalin, glycerol and culture medium with the number of individuals in each life stage group determined as detailed in 3.2.2.1.

Trial 2 - The influence of algal cell concentration on Apocyclops populations

The aim of the trial was to determine the optimal algal cell density required to sustain high density populations of *Apocyclops* when presented a mixed algal species ration.

The six treatment culture media were made up in 0.1 µm filtered seawater at 25 ‰ according to Table 3.2.3 and allowed to equilibrate to 29 °C prior to distribution between the six corresponding replicate culture units. The 1 L reservoir of the previous trial was replaced with a reservoir of 10 L in volume maintained in a 20 L white plastic vessel with vigorous aeration achieved by using an 8 L $hour^{-1}$ flow regulator attached to a 4 mm airline (Figure 3.2.3). The contents of each reservoir were delivered to individual culture units via gravity with discharge rates averaging 300 mL $hour^{-1}$ and were replenished on a daily basis with the respective fresh media.

The thirty-six 500 mL culture units were each inoculated with 10 ovigerous *Apocyclops* isolated directly from the stock culture and rinsed in filtered seawater of 25 ‰ salinity. *Apocyclops* cultures were subsequently treated in a manner similar to that of the previous trial with the exception that temperature, salinity, pH and dissolved oxygen levels of all thirty-six cultures were monitored daily as previous trials indicated that algal cell density exerted a significant influence on all environmental parameters.

Statistical analyses

All environmental parameters and *Apocyclops* data were subjected to Shapiro-Wilk's test for normality and Bartlett's test for homogeneity of variance. Non-normal data were transformed using square root transformation prior to the completion of analyses of variance. Treatment effects in normal data exhibiting homogenous variance were determined by analysis of variance (ANOVA) and Scheffe's multiple means comparison tests.

Data unable to be transformed to meet the underlying assumptions of ANOVA were analysed using the Kruskal-Wallis *k*-sample non-parametric analysis of variance equivalent with differences assessed using a modified Tukey's Test which makes use of sum of ranks as opposed to sum of means (Zar, 1984). All transformed data were converted back into original units of measurement for presentation in supporting figures and tables.

Culture population density data are reported as the equivalent number of *Apocyclops* L^{-1} to enable comparison between trials conducted in culture units of different volumes.

Table 3.2.3: Algal species requirements for cell density trial with *Apocyclops*.

Diet	Total cells mL^{-1}	*Isochrysis* cells mL^{-1}	*Rhodomonas* cells mL^{-1}	*Tetraselmis* cells mL^{-1}
CO	1.0×10^0	0	0	0
C1	5.0×10^3	1,670	1,670	1,670
C2	1.0×10^4	3,340	3,340	3,340
C3	1.6×10^5	53,330	53,330	53,330
C4	3.1×10^5	103,330	103,330	103,330
C5	4.6×10^5	650,000	650,000	650,000

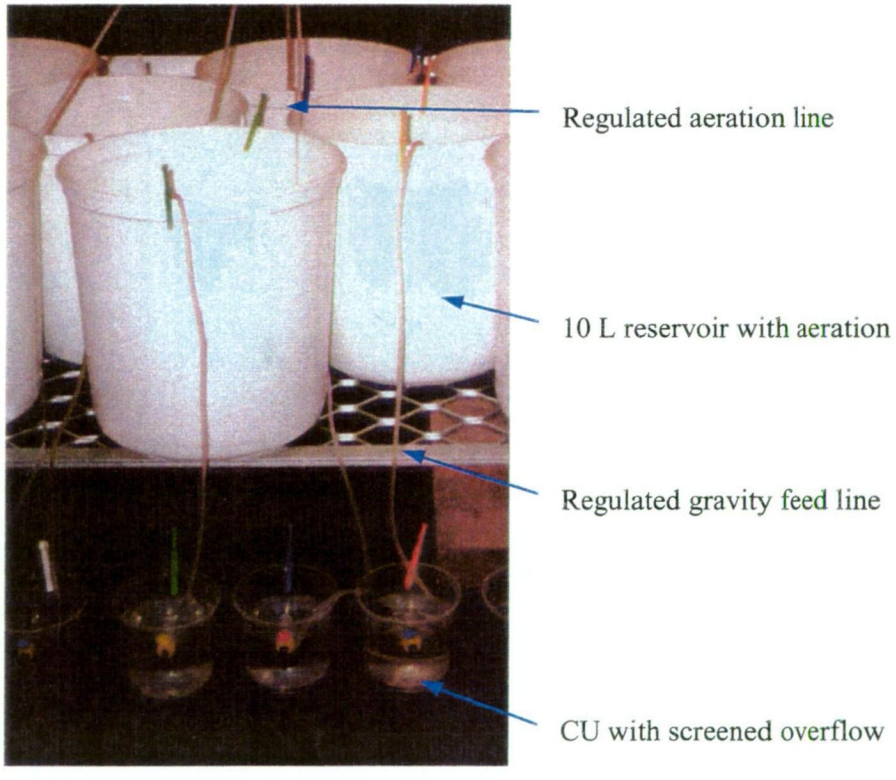


Figure 3.2.3: Photograph of the 500 mL culture units with 10 L reservoirs as used in *Apocyclops* diet trials. Each 500 mL culture unit (CU) is supplied with media from an aerated 10 L reservoir, the contents of which were gravity fed through a 2 mL hr^{-1} dripper into the culture vessel at a rate of 300 mL hr^{-1}. Excess culture media passed through a 63 µm-mesh screened outflow.

3.2.3 *Apocyclops*-fish larvae interaction

The following series of trials were conducted in response to concerns raised by Northern Territory barramundi farmers regarding the potential of *Apocyclops* for piscivory. When shown a photograph of *Apocyclops* the farmers indicated that it was the same copepod they had witnessed 'attack' barramundi larvae in their larval rearing ponds. The identification was made on the basis of the shape of the copepod and the presence of two egg sacs. Other cyclopoid species were also known to be present in the green water ponds, including members of the genus *Oithona* (Appendix B).

The overall aim of the following trials was to assess the nature of any interaction between *Apocyclops* and larval barramundi.

Common methods

Detailed below are the systems, techniques and protocols used to culture and handle the live foods and barramundi larvae used in subsequent trials.

Barramundi larvae production

The barramundi broodstock held by DAC were maintained in a 35,000 litre fibreglass tank operating on a recirculating system under a constant temperature (30 °C), salinity (30 ‰) and photoperiod (13 L:11D) regime. Six female and eight male fish with an approximate biomass of 220 kg were held in the tank. The barramundi were fed a high quality diet comprising freshly thawed whole mullet and squid. Once a week this food was injected with a vitamin mix developed by Queensland Fisheries.

To achieve spawning, female barramundi broodstock yielding 400 µm oocytes in a gonad biopsy sample were hormonally induced using a pellet comprising cholesterol and Luteinising Hormone Releasing Hormone analogue (LHRHa) at a dose rate of 50-100 µg per kg of fish. The females commenced spawning the evening following the day the hormone dose was administered, and they continued to spawn over two to three successive nights. The eggs were released into the holding tank and were automatically concentrated in a floating egg collector comprising a 500 µm screen supported by a square PVC frame. Two airlift systems were used to pass water from the tank through the screen. The morning following a successful spawning, the eggs in the collectors were transferred wet using 180 µm mesh dip nets to $1m^3$ larval rearing tanks filled with 1 µm filtered, ultra-violet sterilised seawater at 30 ‰. Eggs released during the evening took approximately 12 hours to hatch at 30 °C. Approximately 44 hours after hatching, barramundi larvae usually commenced exogenous feeding. Rotifers were added at a density of 10 mL^{-1} on the morning of the second day post-hatch.

Rotifer culture

Small strain rotifers *Brachionus rotundiformus* were maintained on a diet of *Isochrysis* and *Tetraselmis* at 30 ‰ in 1 m^3 tanks supplied with moderate aeration via three weighted 4 mm airlines.

Rotifer culture densities were calculated from counts obtained from three 1 mL samples. The appropriate volume of the culture was removed from the stock culture and the rotifers were rinsed in clean 1 µm filtered, ultra-violet sterilised seawater of 30 ‰ salinity over a submerged 60 µm mesh screen prior to distribution to experimental aquaria.

Commercial enrichment products were not used as the high lipid content of the diet resulted in the rapid deterioration of water quality under the high ambient temperatures, and in previous trials they had delivered little, if any, benefit when compared to rotifers maintained on a mixed *Isochrysis* and *Nannochloropsis* algal diet (Schipp & Pitney, 1995).

Apocyclops culture

Apocyclops stock cultures were maintained in 100 L Nally® bins filled with 1 µm filtered, ultra-violet sterilised seawater at 25 ‰. The cultures were fed a mixture of *Tetraselmis* and *Isochrysis* on a daily basis to maintain algal densities in excess of 10^4 cells mL^{-1}. The cultures experienced temperatures over the range from 28.8 to 33.4 °C.

Apocyclops culture density was calculated from counts obtained from three 10 mL samples. The appropriate number of copepodids were collected over a submerged 60 µm mesh screen and rinsed with clean 1 µm filtered, ultraviolet sterilised seawater at 30 ‰ prior to distribution to individual aquaria.

Water quality assessment

Water quality parameters were monitored at the commencement and completion of each 23-hour trial using methods detailed under Common Methods Section 3.2. The low stocking densities of copepods and fish larvae, in conjunction with the short-term nature of the trials, meant that rigorous testing for levels of nitrogenous compounds was not necessary. Random checks of water, algae or live food cultures were completed to ensure that contamination did not prove a confounding factor in any of the trials. Temperature and salinity were recorded prior to inoculation and at the completion of each 23-hour trial.

3.2.3.1 Copepod piscivory as influenced by density and age of larvae

The following trial was conducted to: a) assess the nature of any interaction between barramundi larvae and *Apocyclops*, b) test the suitability of the mini-aquaria for short-term investigations into the interaction between barramundi larvae

of 3 dph to 6 dph and copepodids of *Apocyclops*, and c) assess the possible impact of handling on barramundi larvae and *Apocyclops*.

Twenty mini-aquaria comprising 250 mL cylindrical clear plastic containers with lids were washed, air-dried, and left filled with seawater for seven days to leach any toxins from the plastic. The mini-aquaria were subsequently filled with 200 mL of 1 µm filtered, ultraviolet sterilised seawater at 30 ‰. After inoculation with copepods and larvae, the mini-aquaria were placed on an elevated bench in an air-conditioned room experiencing a 13 L:11D photoperiod at approximately 360 lux (7.0 µmol $s^{-1}m^{-2}$) at the water surface.

On successive days at 15:00 hours, *Apocyclops* copepodids, ranging from CIII to CV, and barramundi larvae were transferred individually into the mini-aquaria using a wide bore plastic pipette (Ø 3 mm).

Four replicate mini-aquaria were inoculated for each of the five treatments (giving a total of 20 aquaria):

a. 5 barramundi larvae plus 15 *Apocyclops* copepodids,

b. 10 barramundi larvae plus 15 *Apocyclops* copepodids,

c. 20 barramundi larvae plus 15 *Apocyclops* copepodids,

d. 15 barramundi larvae (larval control), and

e. 15 *Apocyclops* copepodids (copepod control).

The larval control and copepod control treatments were included to quantify any confounding effects on barramundi larvae and *Apocyclops* due to handling during inoculation of the trial.

At 14:00 hours on the day following inoculation, the number of barramundi larvae and the number of *Apocyclops* copepodids remaining in each of the twenty culture units were counted.

The inoculum protocol was followed for barramundi of 3, 4, 5 and 6 dph on the day of inoculation. All barramundi larvae were taken from the same batch maintained under consistent environmental and feed conditions (as outlined in the Common methods section 3.2.3).

3.2.3.2 Copepod piscivory as influenced by health and age of larvae

Following the success of the mini-aquaria and handling techniques experienced in the preceding trial, a more comprehensive experimental design was implemented to investigate the influence of larval condition on the propensity of female *Apocyclops* to prey on barramundi. The inclusion of algae and rotifers in the cultures was an

attempt to simulate conditions likely to be encountered in green-water culture systems, where both the barramundi larvae and cyclopoid copepodids would both have a choice of prey items.

On the day exogenous feeding commenced (1 dph), barramundi larvae were transferred to two 100 L Nally® bins at a density of 20 larvae L^{-1}. One batch of larvae was fed a mixture of *Apocyclops* nauplii and rotifers, and the other was left unfed. Prior to inoculation with barramundi larvae and ovigerous *Apocyclops*, twenty-five mini-aquaria were filled with 150 mL of 1 µm filtered seawater at 30 ‰ containing a bloom of *Tetraselmis* (~10^4 cells mL^{-1}) and rotifers at a density of 0.1 mL^{-1}.

At 15:00 hours, five replicate mini-aquaria were inoculated for each of the following treatments (giving a total of 25 aquaria):

a. 10 ovigerous *Apocyclops* – copepod control

b. 10 unfed barramundi larvae – unfed larval control

c. 10 fed barramundi larvae – fed larval control

d. 10 unfed barramundi larvae plus 10 ovigerous *Apocyclops*

e. 10 fed barramundi larvae plus 10 ovigerous *Apocyclops*.

The barramundi larvae in the mini-aquaria were left undisturbed overnight. At 14:00 hours the day following inoculation the number of barramundi larvae and *Apocyclops* copepodids retrieved were recorded. Any differences identified between the number of copepodids or larvae retrieved from mixed treatments compared with those of the corresponding control treatments would theoretically indicate the nature of the interaction between the larvae and the copepods.

Barramundi larvae of 1 dph, 2 dph, 3 dph, 4 dph, 5 dph, 6 dph, and 7 dph were inoculated on successive afternoons with the number of 2 dph, 3 dph, 4 dph, 5 dph, 6 dph, 7 dph and 8 dph barramundi larvae retrieved recorded the day following the each series of inoculations.

Statistical analyses

Differences between the number of copepodids and or larvae retrieved for each treatment were assessed using Analyses of Variance (ANOVA) with Scheffe's multiple means comparisons where data were normally distributed and exhibited homogenous variance as determined by Shapiro-Wilk's and Bartlett's tests respectively. When data did not meet the assumptions of ANOVA, Kruskal-Wallis *k*-sample and Tukey's multiple means comparison tests were used.

3.3 Results

3.3.1 Life cycle and demographics

3.3.1.1 Life cycle

Twelve distinct stages possessing significantly different dimensional and morphological characteristics were identified in the life cycle of *Apocyclops dengizicus*.

Apocyclops eggs, carried in paired eggs sacs on the female urosome, are roughly spherical and approximately 86 µm in diameter. *Apocyclops* nauplii only hatched successfully from egg sacs retained on the female urosome (Figure 3.3.1), and none hatched from discarded egg sacs. Females were found to discard egg sacs as a result of shock from a sudden change of temperature, salinity, rough handling or physical removal. *Apocyclops* females from which egg sacs have been removed, either as a result of shock or by gentle tugging using fine forceps, continue to extrude successive viable egg sacs under favourable conditions.

The six naupliar stages of *Apocyclops* range in length from 114 µm to 335 µm (Table 3.3.1), each differing significantly from each other in both length and width ($p<0.0001$; Figure 3.3.2), and from the succeeding six copepodid stages. The lengths of the six copepodid stages range from 445 µm to 1160 µm (Table 3.3.2). Copepodid stages CIV and CV were not identified in the samples collected, so to provide representative data for the purposes of comparison, growth factor (calculated as the length of the life stage divided by the length of the preceding life stage; Valderhaug & Kewalramani, 1979), was used to estimate the lengths of these two missing life stages.

Apocyclops nauplii are typically pyriform and dorso-ventrally flattened. All naupliar stages are unpigmented except for the red naupliar-eye located anteriorly on the dorsal surface. Lipid droplets are occasionally visible in the body of the nauplius, and pigmentation of gut contents is also visible on occasions. The increase in size and change in shape with successive developmental stages is noticeably distinct. Metamorphosis from NVI to CI is associated with elongation of the body and the appearance of metasomal and urosomal differentiation (Figure 3.3.3). The *Apocyclops* copepodid is much broader in the cephalothoracic region than the urosome, with two pairs of periopods borne on the first two thoracic segments.

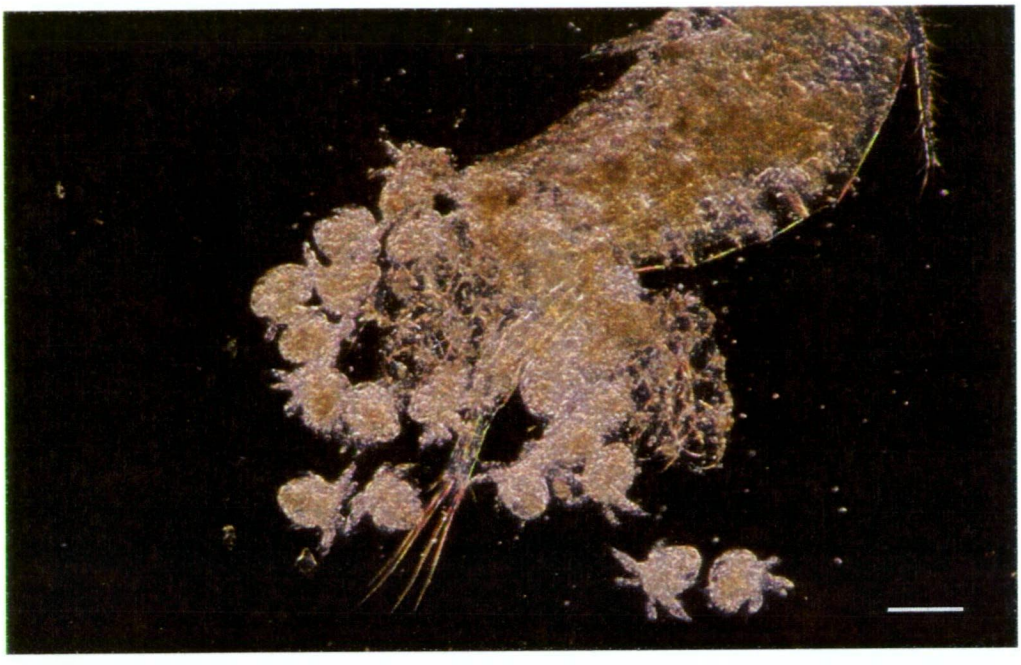


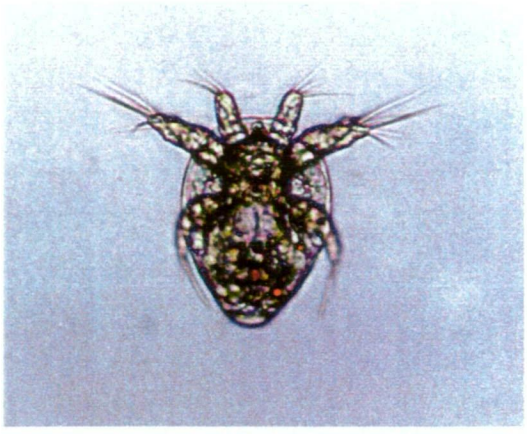
Figure 3.3.1: *Apocyclops* nauplii hatching from the egg sac while still attached to the female urosome. Scale bar is 100µm.

Table 3.3.1: Dimensions (mean ±SE) of the six naupliar stages of *Apocyclops* where L corresponds to nauplius length as measured from the anterior to the posterior of the nauplius body, and W to cephalosome width at the widest point. L:W is the ratio of length to width. SE indicates standard error, and n sample size.

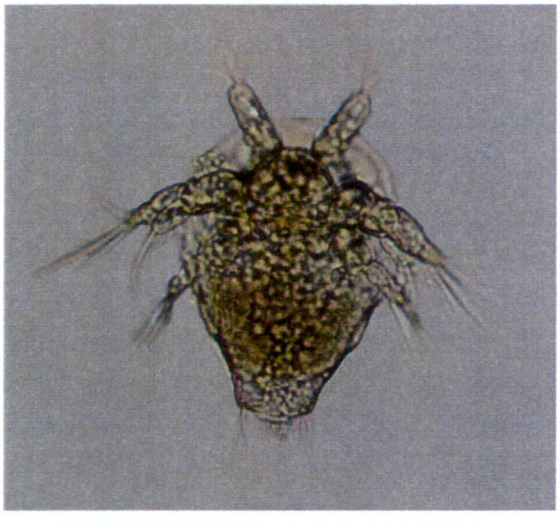
Life Stage	L ±SE (µm)	W ±SE (µm)	n	L:W
N I	114 ±3	91 ±3	10	1.3
N II	169 ±4	119 ±2	16	1.4
N III	191 ±3	138 ±3	17	1.4
N IV	248 ±5	171 ±2	38	1.5
N V	277 ±1	183 ±2	50	1.5
N VI	335 ±2	195 ±2	9	1.7

Table 3.3.2: Dimensions (mean ±SE) of the six copepodid stages of *Apocyclops* where L corresponds to total body length measured from the rostrum to the last urosomal segment, and W to cephalosome width at the widest point. L:W is the ratio of length to width. SE indicates standard error, and n sample size. * estimated using a calculated growth factor increment (Valderhaug & Kewalramani, 1979).

Life Stage	L ±SE (µm)	W ±SE (µm)	n	L:W
C I	445 ±4	198 ±3	13	2.3
C II	577 ±10	223 ±3	13	2.6
C III	667 ±8	242 ±9	15	2.8
C IV	770*	255*	-	3.0
C V	890*	270*	-	3.3
C VI male	915 ±9	277 ±3	45	3.3
C VI female	1160 ±16	368 ±6	17	3.2

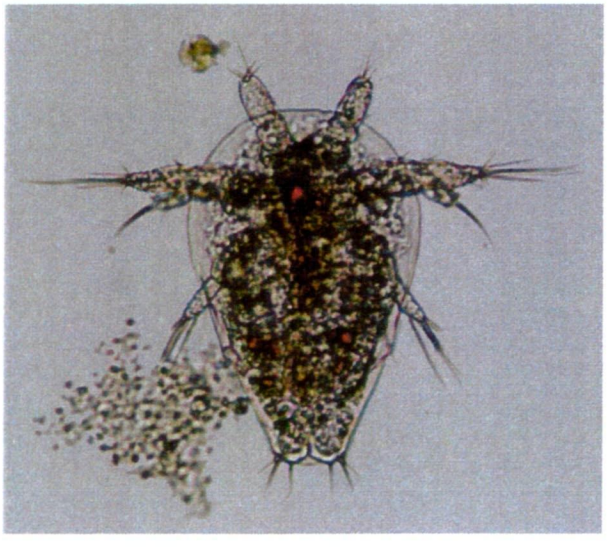


NII



Scale bar

NIV



NVI

Figure 3.3.2: Photomicrographs of representative nauplius stages of the Northern Territory *Apocyclops dengizicus*. Scale bar is 100µm.

Figure 3.3.3: Photograph illustrating the change in shape associated with metamorphosis from nauplius to copepodid stage of the Northern Territory isolate of *Apocyclops dengizicus*. Scale bars is 100 µm

Development through the first five copepodid stages is associated with a progressive increase in segmentation, appearance of three additional pairs of swimming legs and gender-specific specialisation of the urosome and antennules. Sexual dimorphism is obvious at CVI with adult female *Apocyclops* 27% larger than adult males. Both antennules of male *Apocyclops* are stout, geniculate and distinctive compared with the slender and non-geniculate antennules of the female (Figure 3.3.4).

Analyses of variance of length data revealed a highly significant difference ($p<0.0001$) between life stages identifying length as a useful characteristic by which to differentiate between the life stages of *Apocyclops*. Initial identification of the developmental stage of nauplii and copepodids based on length may be further qualified through the use of easily discernable morphological differences associated with the life stages of cyclopoid copepods and more specifically descriptions provided by Valderhaug & Kewalramani (1979).

Figure 3.3.4: Photomicrographs illustrating sexual dimorphism in the antennules of copepodid CVI of *Apocyclops dengizicus*.
(a) males antennules
(b) female antennules

3.3.1.2 Demographics and productivity

A mean generation time (T) of 5.3 days was observed for *Apocyclops* maintained under a temperature range from 28.5 °C to 31.6 °C with a mean of 30.5 °C. The duration of each life stage is approximately 9 hours with egg sac production requiring an additional day, reflecting the 24 hour monitoring increment.

Female fecundity ranged from 3 to 43 copepodids surviving per female corresponding to a mean of 20 individual progeny maturing per female. The number of progeny reaching maturity was observed to decrease over successive egg sacs (Figure 3.3.5). Net reproductive rate (R_o) was calculated as 5.6 ±0.7, however the parameter was highly variable ranging from R_o of 1 through to 38. Table 3.3.3 provides a summary of the demographic information collected.

Table 3.3.3: Demographic information collected for *Apocyclops* when maintained at 30 °C and 30 ‰ on a diet of *Tetraselmis* and *Isochrysis*.

Parameter	F_0
Female longevity (days)	13.3 ±1.3
Egg sacs per female	4.2 ±0.48
% egg sacs hatch	100
% hatched egg sacs maturing	96
% aborting as nauplii	4
Net rate of reproduction (R_0)	5.6 ±0.69
Mean generation time (T)	5.3 ±0.09
Sex Ratio	1.74 ±0.29
% F_0 producing F_1 females	96
% F_1 producing F_2 females	86

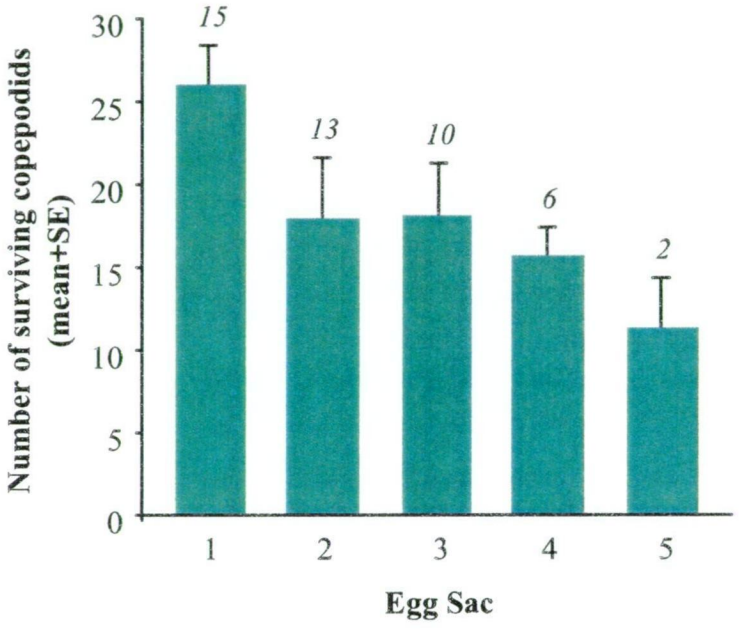


Figure 3.3.5: Average number of progeny (mean +standard error) reaching maturity from successive egg sacs produced by the same *Apocyclops* female. The italicised superscripts indicate respective sample sizes (i.e. the number of egg sacs from which progeny developed).

3.3.2 Culture of *Apocyclops*

3.3.2.1 The effect of salinity

Trial 1 – Tolerance of Apocyclops *to rapid salinity change*

Ovigerous *Apocyclops* were observed to tolerate salinity increments of 20 ‰. Nauplii were observed to develop normally at all nine salinities ranging from 10 ‰ to 50 ‰.

The fact that all females had retained their egg sacs on the day following transfer indicated that the rinsing process followed by transfer to the treatment salinities did not adversely affect their brooding capabilities.

Completion of one-way ANOVA and repeated measures ANOVA failed to identify any significant differences as a result of the salinity treatments. No significant salinity effect ($p>0.05$) was identified overall, or at any of the four observation times on days 1, 3, 5 and 7 of the trial. However, the proportion of *Apocyclops* females carrying egg sacs declined significantly across all salinities over the seven-day period from 100% to 45.6% (Table 3.3.4).

Table 3.3.4: The influence of time on the combined proportion of *Apocyclops* females carrying eggs after 1, 3, 5 and 7 days exposure to salinities ranging from 10 to 50 ‰. Different scripts indicate significant differences ($p<0.05$) as determined by ANOVA of square root transformed count data and Scheffe's multiple means comparisons.

Time (days)	% Ovigerous (mean ±SE)	n	$p<0.05$
1	100 ±0	18	*a*
3	67.8 ±16.0	18	*b*
5	42.2 ±10.0	18	*bc*
7	45.6 ±10.7	18	*c*

Trial 2 - The effect of salinity in non-aerated Apocyclops *cultures*

Apocyclops cultures maintained at 20 ‰ yielded the most dense populations corresponding to an average of 2,406 ±219 individuals L^{-1}. *Apocyclops* cultures maintained at higher salinities between 35 and 50 ‰ produced significantly fewer individuals than those maintained over the range 10 to 30 ‰ (Figure 3.3.6).

The number of *Apocyclops* individuals collected in the replicate 50 mL samples every third day of the trial exhibited an upward trend over time (Table 3.3.5). A significant increase in culture density was observed after nine days ($p<0.001$). An average population density equivalent to 2,260 ±215 *Apocyclops* L^{-1} was present in cultures after three weeks across all eighteen combinations encompassing the ten replicates at each of the nine salinities. The maximum individual culture density achieved after 21 days from an inoculum of five ovigerous females was equivalent to 9,560 individuals L^{-1}.

Table 3.3.5: The trend of increasing *Apocyclops* population density (mean ±standard error) with time across salinities from 10 ‰ to 50 ‰. Different scripts indicate significant differences (p <0.05) identified by repeated measures ANOVA and Scheffe's multiple means comparison test using square root transformed data.

Time (days)	*Apocyclops* L^{-1} (mean ±SE)	maximum density L^{-1}	$p<0.05$
3	237 ±17.1	740	a
6	251 ±223	1040	*ab*
9	1800 ±1530	6200	*bc*
12	2100 ±1340	6640	*bc*
15	1790 ±1140	4840	*ac*
18	1430 ±871	4680	*ac*
21	2500 ±1310	9560	*bc*

No significant difference was detected between the sex ratios of *Apocyclops* populations maintained at each of the seven salinities on either sampling day. The mean sex ratio across all salinities on days 9 and 21 were 2.1 ±0.21 and 2.3 ±0.16 respectively, and they were found not to differ significantly from each other when analysed using the non-parametric Mann-Whitney *U*-test.

An indicative R_o was calculated on the basis that a 50 mL sample from 500 mL inoculated with 5 ovigerous *Apocyclops* corresponds to an initial female density of 0.5 females per 50 mL sample. Populations maintained at 20 ‰ exhibited the greatest indicative R_0 of 15.7 ±3.74 which did not differ significantly from that

calculated for populations at 15 ‰ and 25 ‰ which showed significantly greater R_0 than *Apocyclops* populations cultured at 10 ‰ and between 30 ‰ and 50 ‰ (Figure 3.3.7).

Using the numbers of females observed on days 9 and 21, an average indicative R_0 of 9.3 ±1.3 was calculated for *Apocyclops* populations for day 21 which did not differ significantly from the mean indicative R_0 calculated for populations on day 9 of 9.3 ±0.9. The influence of salinity on indicative R_0, although significant after 9 days culture, was insignificant after 21 days culture ($p>0.05$) (Figure 3.3.7). No interaction was identified between salinity and time factors.

The mean temperature recorded was 31.5 ±0.03 °C (ranging from 31.3 to 31.9 °C), and did not differ significantly between water baths ($p>0.05$). Similarly pH, ammonia (mg unionised NH_3 L^{-1}) and nitrite (mg NO_2 L^{-1}) levels recorded for the exchange media at each salinity did not differ between replicate cultures maintained in different water baths. The media made up on consecutive days over which the 80% media exchanges were conducted were considered similar. Nitrite was absent from the fresh media made up on the twelve occasions, and the unionised ammonia levels recorded for the media at the nine salinities also failed to differ, being present only at trace levels.

The treatment salinity levels were found to differ significantly ($p<0.01$) as did the pH levels of the media. However, only freshly prepared media at 30 ‰ and 50 ‰ differed from each other, the remaining seven media at different salinities exhibited similar pH levels. The mean pH of media at 30 ‰ was 8.3 ±0.04 and 8.7 ±0.02 at 50 ‰, a mean difference of 0.4 units - a difference unlikely to be of biological significance. The pH of all media varied by 0.7 pH units from 8.0 to 8.7. At the end of each of the three-day sampling periods and the end of the 21-day trial period, all cultures exhibited similar pH, ammonia and nitrite levels, the means and ranges are presented in Table 3.3.6.

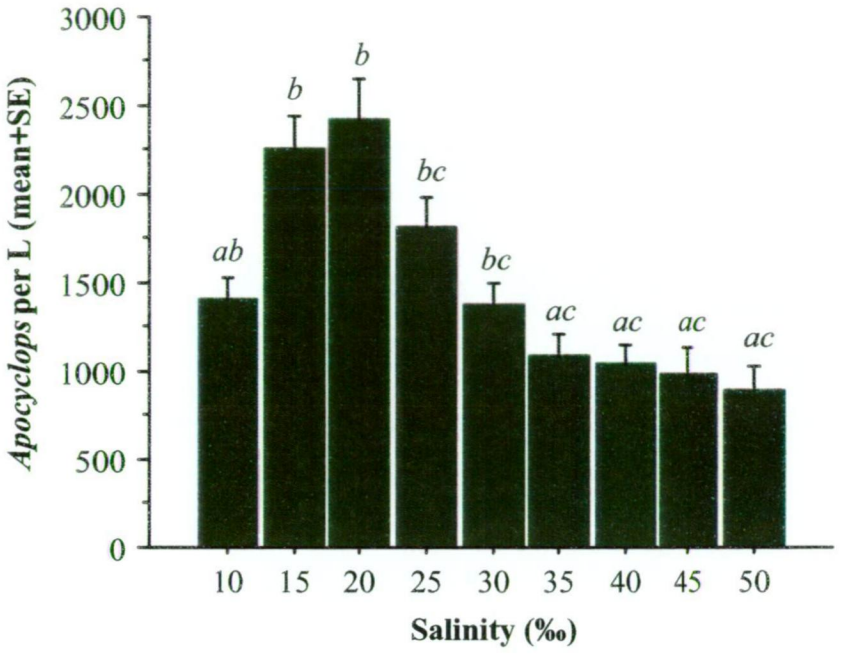


Figure 3.3.6: Effect of salinity on culture productivity as indicated by the total number of *Apocyclops* developing over three weeks. Italicised superscripts indicate significant differences identified by a repeated measures ANOVA and Scheffe's multiple means comparison test conducted using square root transformed data collected at three day intervals.

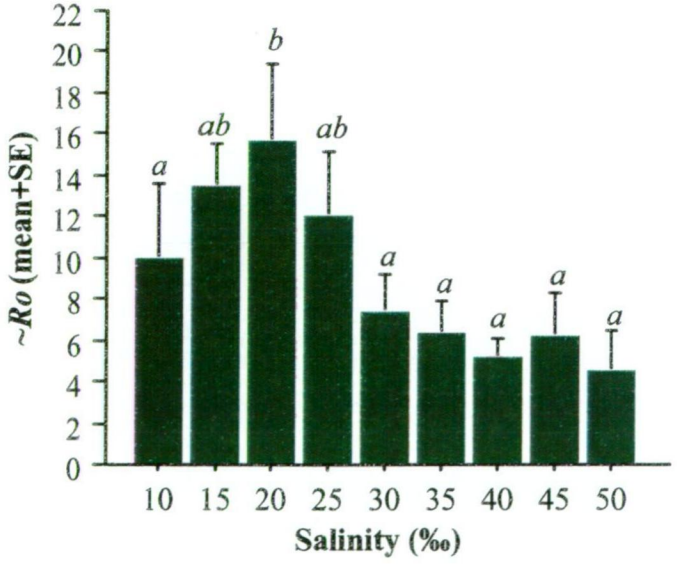


Figure 3.3.7: Influence of salinity and time on the indicative net reproductive rate ($\sim R_0$) of *Apocyclops* cultures maintained at seven salinities: 10, 15, 20, 25, 30, 35, 40, 45 and 50 ‰ after nine days in culture. Italicised superscripts indicate significant differences ($p<0.05$) identified by ANOVA and Scheffe's multiple means comparison test.

Table 3.3.6: Summary data for pH, ammonia and nitrite levels, means (±standard error) and ranges for cultures maintained at salinities ranging from 10 to 50 ‰.

Treatment	Salinity (‰)	pH	Ammonia $mgNH_3\ L^{-1}$	Nitrite $mgNO_2\ L^{-1}$
10	10.0 ±0.00	7.88 ±0.048	0.04 ±0.005	0.12 ±0.042
15	15.0 ±0.00	7.95 ±0.029	0.05 ±0.003	0.14 ±0.017
20	20.0 ±0.00	8.00 ±0.000	0.06 ±0.007	0.26 ±0.103
25	24.8 ±0.14	8.05 ±0.029	0.06 ±0.002	0.17 ±0.073
30	30.9 ±0.31	8.05 ±0.029	0.06 ±0.003	0.22 ±0.104
35	35.8 ±0.25	8.08 ±0.025	0.06 ±0.011	0.40 ±0.191
40	39.8 ±0.25	8.13 ±0.025	0.05 ±0.008	0.19 ±0.083
45	45.0 ±0.00	8.05 ±0.050	0.05 ±0.006	0.17 ±0.079
50	49.8 ±0.48	8.00 ±0.041	0.04 ±0.004	0.46 ±0.241

Trial 3 -. The effect of salinity in flow-through Apocyclops cultures

The most dense *Apocyclops* cultures were those maintained at 20 ‰ salinity. After nine days, the average number of *Apocyclops* individuals developing from an inoculum of ten ovigerous *Apocyclops* was 326 ±15 per culture corresponding to 656 individuals L^{-1} (Table 3.3.7). Cultures maintained at salinities from 15 to 30 ‰ yielded similar numbers of copepods, with cultures maintained at 20 and 25 ‰ salinity yielding significantly more individuals than those developing at 35 ‰.

No significant difference ($p>0.05$) was detected between the numbers of females removed from the five salinities on day 4 of the trial. An average of 9.3 ±0.52 females were removed from each replicate. The across treatment mean of 0.7 females which were not recovered may be attributed to natural mortality over the four days.

The effect of salinity on population structure, sex ratio (F:M) and an indicative net rate of reproduction ($\sim R_0$) were calculated. The data were unable to be transformed to meet the assumptions of ANOVA, so they were analysed using Kruskal-Wallis k-sample test. No significant differences were detected between the sex ratios or $\sim R_0$ exhibited by cultures maintained at the five salinities. The mean sex ratio was 3.4 ±0.39 and indicative R_0 values producing a mean of 16.7 ±1.0 (Table 3.3.8).

Salinity did not appear to exert any influence on *Apocyclops* size. No significant differences were evident in body length, cephalosome length or egg size between treatments (Table 3.3.9). However, a significant difference ($p<0.05$) was detected in the numbers of eggs recorded per egg sac. At 25 ‰ and 30 ‰ salinity,

ovigerous *Apocyclops* produced thirty percent more eggs than those at 15, 20 and 35 ‰ salinity (Figure 3.3.8).

Temperature and light variations were experienced across the treatments but were not significant, the mean temperature being 29.1 ±0.005 °C (mean ±SE) and the mean light level across treatments being 52 ±0.6 lux (mean ±SE).

Significant differences ($p<0.05$) were detected for pH and dissolved oxygen (Table 3.3.10). Although statistically different, the absolute range experienced in relative terms is insignificant biologically, with pH ranging from 7.8 to 8.0, and dissolved oxygen from 82% to 90%.

Table 3.3.7: Mean (±standard error) and range of *Apocyclops* culture densities (individuals L^{-1}) as influenced by salinity at 15, 20, 25, 30 and 35 ‰ over nine days. Scripts indicate significant differences ($p<0.05$) identified by ANOVA using square root transformed data and Fisher's protected least significant difference means comparison.

Salinity (‰)	*Apocyclops* L^{-1} (mean ±SE)	Minimum	Maximum	$p<0.05$
15	540 ±50	360	400	*ab*
20	655 ±30	610	735	*b*
25	605 ±30	535	668	*b*
30	465 ±30	400	535	*ab*
35	400 ±85	145	500	*a*

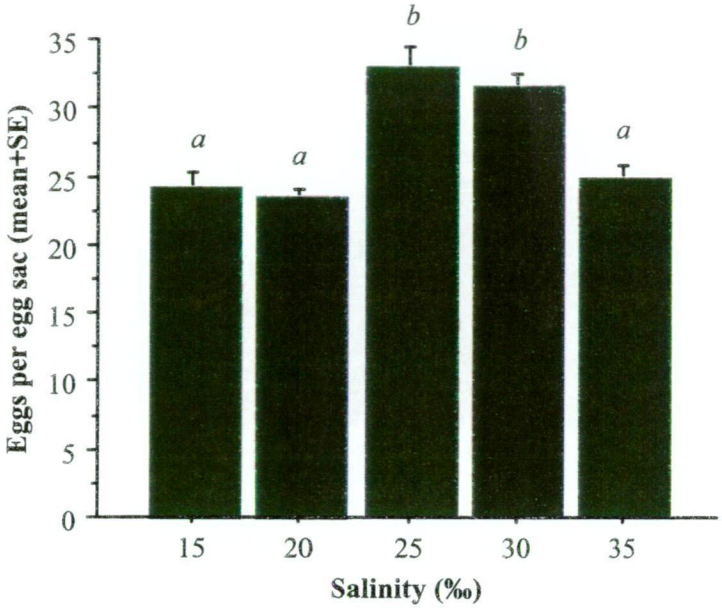


Figure 3.3.8: Influence of salinity on the number of eggs (mean +standard error) per pair of ovigerous *Apocyclops* egg sacs when maintained in flow-through systems for nine days. Italicised superscripts indicate significant differences ($p<0.05$) as determined by ANOVA and Scheffe's multiple means comparison using sqaure root transformed data.

Table 3.3.8: Estimated net rate of reproduction (R_0 mean ±standard error) for *Apocyclops* cultures maintained at five salinities for nine days. No significant differences were identified ($p>0.05$).

Salinity	Mean G3	Mean G2	R_0
15 ‰	160	8.3	19.6 ±1.24
20 ‰	179	9.0	20.3 ±2.39
25 ‰	149	8.5	18.9 ±4.20
30 ‰	138	9.8	15.5 ±2.59
35 ‰	154	7.8	18.5 ±7.57

Table 3.3.9: Mean *Apocyclops* body length, cephalosome length and egg diameter averaged across all five salinity treatments. No significant salinity effects were detected. SE – standard error, n – sample size.

Measurement	Mean	SE	n
Body length (µm)	1067	5.28	150
Cephalosome length (µm)	350	18.0	150
Egg diameter (µm)	89.3	2.54	150

Table 3.3.10: Dissolved oxygen and pH levels recorded when assessing the influence of salinity on *Apocyclops* culture productivity over the range of 15 ‰ to 35 ‰.

Salinity	Dissolved Oxygen (%)		pH	
	mean ±SE	$p<0.05$	mean ±SE	$p<0.05$
15 ‰	82 ±0.3	*a*	7.97 ±0.03	*a*
20 ‰	86 ±1.0	*ab*	7.90 ±0.00	*ab*
25 ‰	88 ±0.3	*b*	7.86 ±0.03	*ab*
30 ‰	88 ±1.5	*b*	7.90 ±0.00	*ab*
35 ‰	88 ±0.9	*b*	7.80 ±0.00	*b*

3.3.2.2 The effects of temperature, diet, aeration and handling

Temperature, diet and aeration all exerted significant influences on *Apocyclops* culture population density ($p<0.05$), but handling effects were not significant. The maximum *Apocyclops* density was observed in cultures maintained at 32 °C without aeration when fed a diet of *Tetraselmis* at 1-2 x10^4 cells mL^{-1} yielding 666 individuals from 20 ovigerous *Apocyclops* after 14 days corresponding to a population density of 1,335 individuals L^{-1} (Figure 3.3.9).

Higher temperatures encouraged greater productivity than lower temperatures with 29 °C, 32 °C and 35 °C yielding *Apocyclops* densities of approximately 500 individuals L^{-1} (Table 3.3.11).

Apocyclops cultures fed microalgal diets produced more dense populations than those cultures fed fish crumble. Cultures fed on *Tetraselmis* yielded an average density equivalent to 612 ±139 *Apocyclops* L^{-1} compared with 328 ±76 individuals L^{-1} developing on a diet of *Isochrysis* which did not differ significantly from population densities of cultures that were fed fish crumble (187 ±47 *Apocyclops* L^{-1}).

Analysis of the combined influences of temperature and diet on *Apocyclops* population growth identified *Tetraselmis* as the most productive diet over the three higher temperatures. Trends suggest that 32 °C is the optimum culture temperature, supported by the results from the analysis of the interaction between temperature and aeration.

The negative influence of aeration on *Apocyclops* culture density was also significant ($p<0.05$), with aerated cultures yielding 45 percent less *Apocyclops* (205 ±25 individuals L^{-1}) compared with the average yield from non-aerated cultures of 375 ±35 individuals L^{-1}.

The interaction between temperature, diet and aeration on *Apocyclops* productivity suggests that greatest *Apocyclops* culture productivity is achievable from cultures maintained without aeration on a diet of *Tetraselmis*, between 29 and 32 °C when fed every four days (Figure 3.3.9).

The final factor to be assessed was handling. Due to the presence of the interaction between temperature, diet and aeration factors, the residuals of the means for all combinations were derived leaving behind the residual effect attributable to handling treatment. Analyses of the residuals revealed no significant difference in *Apocyclops* population density attributable to the handling treatment *per se*.

The influence of monitoring on productivity was negligible which is not surprising, as *Apocyclops* has proven a hardy copepod capable of withstanding partial desiccation, and thermal and salinity shocks. The application of the handling treatment coincident with the exchange of culture media for all culture units may have masked the effects of the treatment factor.

Recorded treatment temperature levels differed significantly (Table 3.3.11), with no block effect evident ($p>0.05$). All cultures experienced a mean salinity of 30.1 ±0.08 ‰ ranging from 29 to 34 ‰.

No significant differences in pH were identified between treatments when data collected were grouped by temperature or diet for pH, dissolved oxygen and ammonia levels ($p>0.05$). However significant differences ($p<0.0001$) were identified when the same data was grouped according to aeration. Aerated cultures exhibited mean pH level of 8.2 ±0.02, mean dissolved oxygen levels of 93.6 ±0.64% compared with a mean pH of 7.8 ±0.03 and mean dissolved oxygen levels of 78.0 ±1.95% for non-aerated cultures (Table 3.3.12).

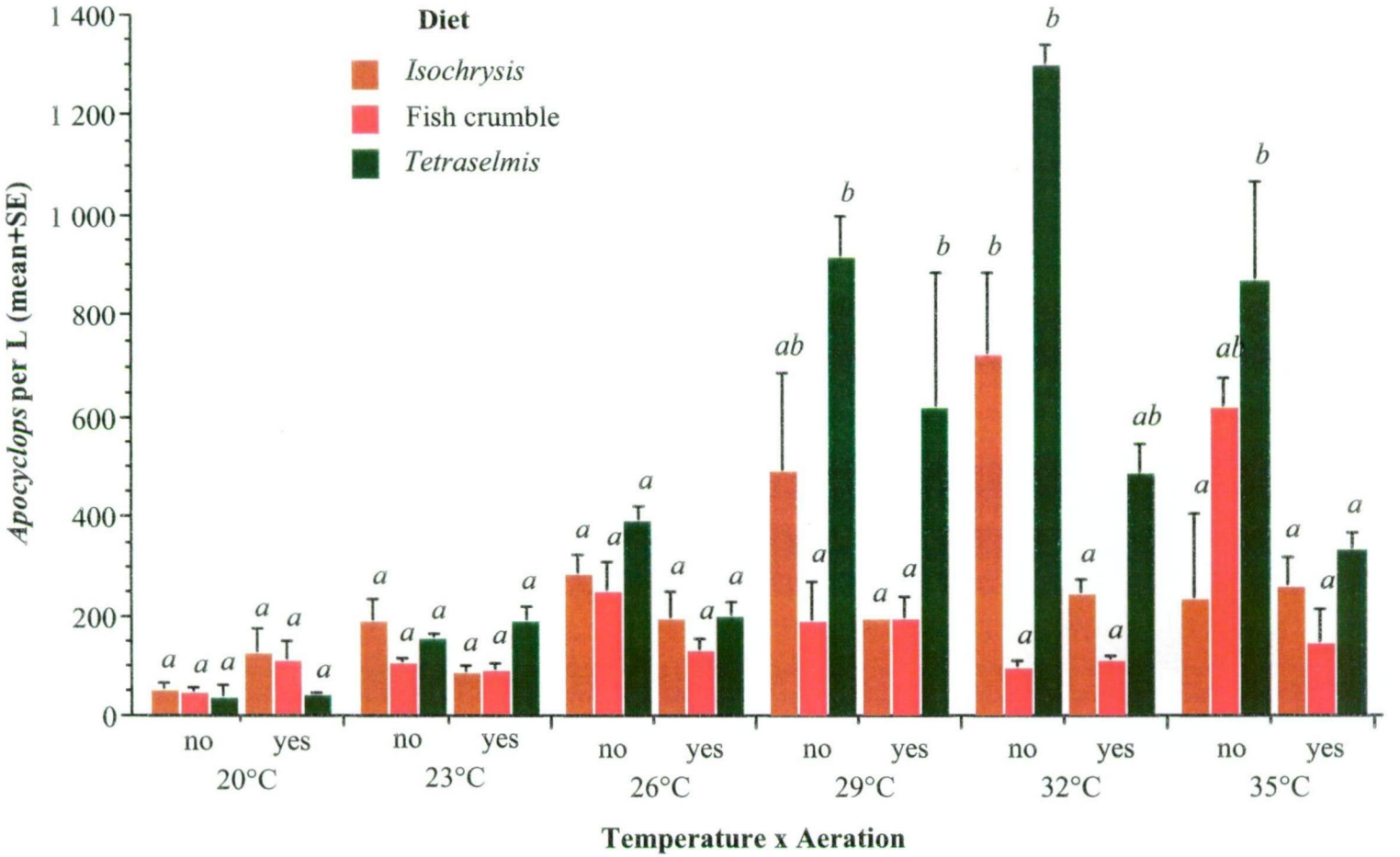


Figure 3.3.9: The combined influence s of temperature, diet and aeration on the productivity of *Apocyclops* cultures as indicated by the total number of individuals developing over a 14 day period. Scheffe's comparison of means was conducted on square root transformed data. Italicised superscripts indicate significant differences ($p<0.05$)

Nitrite levels recorded did not differ significantly ($p<0.05$) for data grouped according to temperature or aeration. However diet treatment did influence nitrite levels. *Tetraselmis* fed cultures nitrite levels were significantly lower (1.1 ±0.42 mg NO_2 L^{-1}) than those recorded for *Isochrysis* and fish crumble fed diets (3.3 ±0.49 mg NO_2 L^{-1}).

Table 3.3.11: Average temperature and population density (mean ±standard error) for each of six temperatures. Different scripts indicate significant differences ($p<0.05$) identified by one-way ANOVA and Scheffe's comparison of means using square root transformed population data.

Treatment	Temperature (°C)	*Apocyclops* L^{-1}	$p<0.05$
20 °C	20.0 ±0.03	67.2 ±12.9	*a*
23 °C	23.1 ±0.03	134 ±14.8	*ab*
26 °C	26.1 ±0.03	240 ±27.2	*ab*
29 °C	29.1 ±0.03	434 ±92.4	*b*
32 °C	32.0 ±0.03	492 ±130	*b*
35 °C	35.0 ±0.03	368 ±84.8	*b*

Table 3.3.12: The mean (±standard error) levels of pH, dissolved oxygen (DO), ammonia and nitrite for *Apocyclops* cultures either without aeration or with aeration, when maintained under varying conditions of temperature and diet.

Parameter	no air	with air	*p*-value
pH	7.86 ±0.026	8.15 ±0.016	<0.05
mg unionised NH_3	0.13 ±0.030	0.14 ±0.035	<0.05
mg O_2 L^{-1}	5.5 ±0.13	6.4 ±0.09	<0.05
% O_2	78 ±2.0	94 ±0.6	<0.05
salinity ‰	30.3 ±0.13	30.1 ±0.08	>0.05
mg NO_2 L^{-1}	2.0 ±0.45	1.5 ±0.41	>0.05

3.3.2.3 The effect of temperature

Temperature exerted a significant influence on *Apocyclops* culture productivity, as indicated by the final total number of individuals developing over the three week period in mass culture units. The optimum culture temperature occurred over the range 26 and 32 °C resulting in an average density equivalent to 255 individuals L^{-1} (105 to 440 *Apocyclops* L^{-1}; Figure 3.3.10).

The influence of temperature on population composition assessed using non-parametric analyses failed to identify any significant effects ($p<0.05$) on the relative proportions of ovigerous females, adults, immature copepodids, or nauplii. Ovigerous *Apocyclops* and nauplii represented less than 2% of the total population; immature copepodid stages (CI-CV) comprised 80% of the population, and adults 18%.

Population composition at different temperatures did identify significant differences ($p<0.01$) in terms of the numbers of adults and immature copepodids recorded, with no significant difference in the numbers of ovigerous *Apocyclops* or nauplii present (Figure 3.3.11).

After three weeks of culture the average density of ovigerous *Apocyclops* was 3.2 L^{-1}, ranging from 0 to 15 L^{-1}. Similarly, naupliar numbers were low with a mean of 5.2 L^{-1}, ranging from 0 to 55 L^{-1}. Immature copepodid densities were higher with a mean of 30 L^{-1} ranging from 0 to 75 L^{-1}. The dominant stage in cultures after this time were adults that exhibited a mean of 165 L^{-1} and an associated density range from 20 to 390 copepodid stage CVI L^{-1}.

The greatest mean adult *Apocyclops* densities were recorded from cultures maintained between 26 °C and 32 °C corresponding to 225 adult *Apocyclops* L^{-1}. The demographic composition of cultures maintained at 23 °C reflects the effect of slowed metabolic rate on development with the immature stages being more abundant (Figure 3.3.12). The influence of temperature on instar duration is evident with a reduced duration with increasing temperature explaining compositional differences.

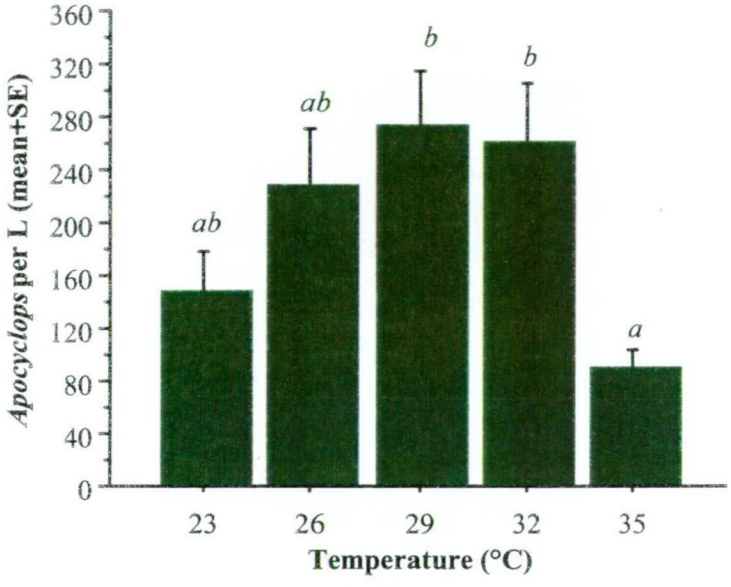


Figure 3.3.10: Influence of temperature on *Apocyclops* productivity over the temperature range 23 to 35 °C as indicated by the total number of individuals developing in cultures over three weeks. Italicised superscripts indicate significant differences ($p<0.05$) as determined by ANOVA, conducted using square root transformed data, and Scheffe's multiple means comparisons.

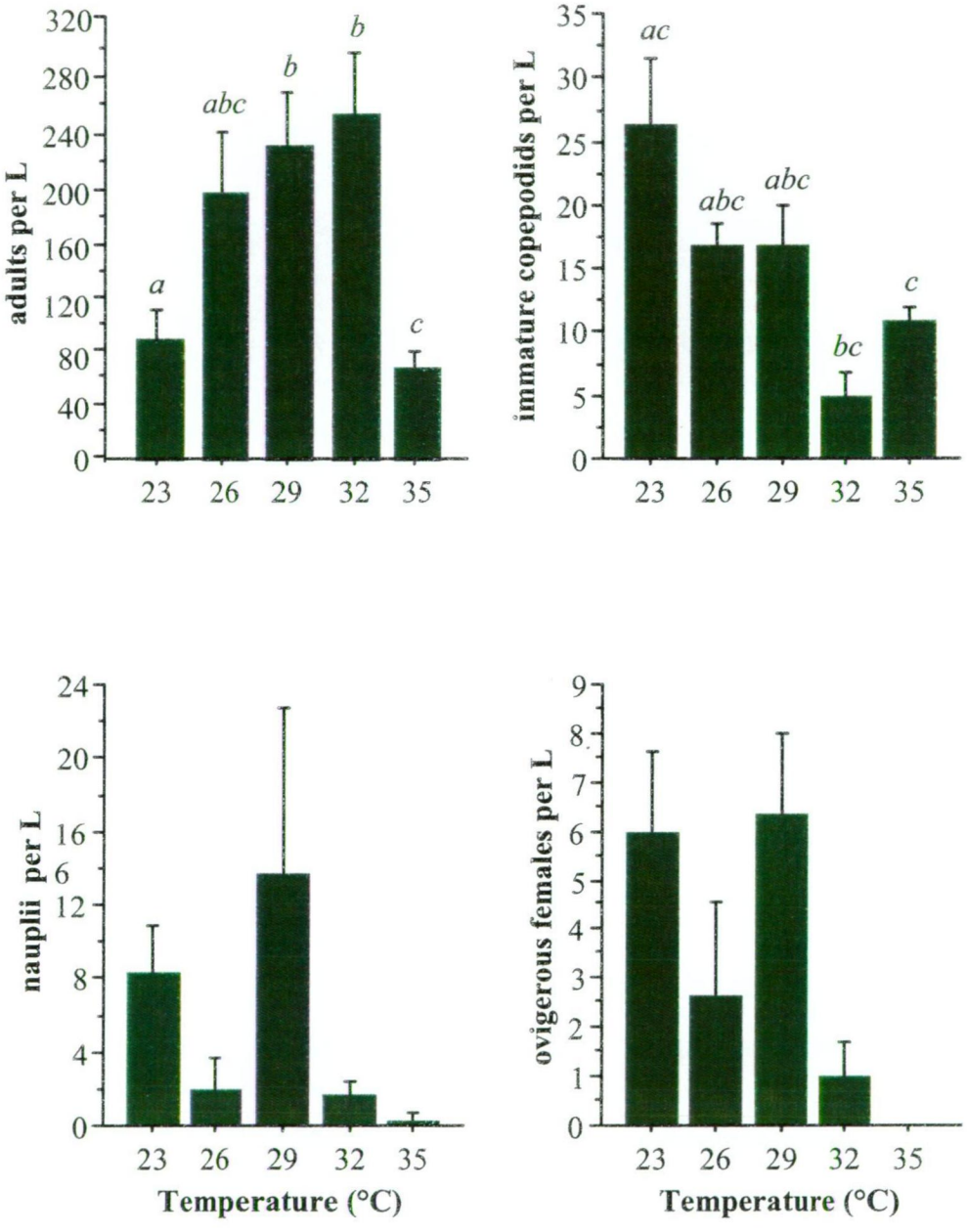


Figure 3.3.11: *Apocyclops* population composition (numbers within demographic groups) when cultured at different temperatures for three weeks. Italicised superscripts indicate significant differences ($p<0.01$) as determined by ANOVA of arcsine square root transformed data and Scheffe's multiple means comparison.

Information recorded from individual 20 mL cultures enabled representative mean generation times to be calculated. Only progeny derived from females maintained at 29 °C developed through to ovigerous females. The majority of the progeny from 23 °C failed to develop through to maturity and reproduce during the three-week trial. As a result, analyses were conducted using an estimate of the mean generation time ($\sim T$) corresponding to the time required to develop from nauplius stage one (NI) through to copepodid stage six (CVI). The curvilinear relationship between $\sim T$ and culture temperature enabled calculation of $\sim T$ for 26 °C and 32 °C (Figure 3.3.12). $\sim T$ corresponding to cultures maintained at 32 °C and 35 °C were the shortest, with those corresponding to cultures at 23 °C being 35% longer (Table 3.3.13).

The indicative net rate of reproduction ($\sim R_0$) calculated as the ratio of the number of inoculum females to the total number of females developing from their respective egg sacs, revealed that cultures maintained at 32 °C exhibited the highest $\sim R_0$ of 7.0 ±1.1. The $\sim R_0$ of *Apocyclops* cultures at 32 °C were significantly different to those of cultures maintained at 23 °C and 35 °C (2.5 ±0.5 and 2.1 ±0.4 respectively), but did not differ significantly from the $\sim R_0$ calculated for cultures at 26 °C and 29 °C (4.8 ±0.7 and 5.7 ±1.1 respectively).

For each temperature, an indicative intrinsic rate of natural increase ($\sim r$) was calculated using the formula: $r = (\ln R_0) / T$ combining the observed values of $\sim R_0$ and the calculated $\sim T$ (Milou & Moraitou-Apostolopoulou, 1991a). Once again, *Apocyclops* cultures maintained at 32 °C yielded the highest r (0.24 ±0.02) being significantly different from those maintained at 23, 26 and 35 °C (0.07 ±0.02, 0.15 ±0.02 and 0.09 ±0.03 respectively), but not differing from that of cultures maintained at 29 °C (0.18 ±0.02).

The sex ratio of *Apocyclops* (female CVI: male CVI) was not significantly affected by temperature over the range investigated. The mean sex ratio was 1.6 ±0.41, ranging from 0.29 to 13.3. Generally mean sex ratios for culture populations at each temperature were greater than 1.0 (Table 3.3.14).

The temperature ranges recorded for the three water baths calibrated at each of the five temperatures were similar ($p>0.05$) exhibiting average values within 0.1 °C of the target temperature and a standard error of 0.03 °C.

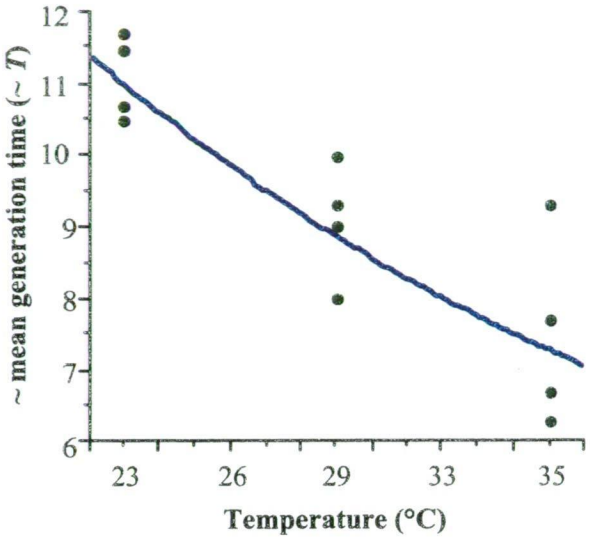


Figure 3.3.12: Mean generation time of *Apocyclops* where ~*T* is an estimate of the mean generation time corresponding to the time for nauplius stage one (NI) to develop through to copepodid stage six (CVI). The significant ($p<0.001$) relationship is represented by the equation $\sim T = 23.924 - 0.729*T°C + 0.007* T°C^2$.

Table 3.3.13: Mean generation time (~*T*) of *Apocyclops*. Actual values are the average observed *T* for cultures maintained at 23, 29 and 35 °C, estimated values being those derived from a polynomial regression $\sim T = 23.924 - 0.729*°C + 0.007* °C^2$ ($R^2 = 0.763$, Figure 3.3.12).

Temperature	Actual ~*T* (mean ±SE)	Calculated Estimate ~*T*
23 °C	11.0 ±0.26	10.86
26 °C	-	9.70
29 °C	8.9 ±0.39	8.67
32 °C	-	7.76
35 °C	7.3 ±0.57	6.98

Table 3.3.14: Mean sex ratio (female CVI: male CVI) exhibited by *Apocyclops* culture populations maintained at temperatures of 23, 26, 29, 32 and 35 °C. No significant differences were identified ($p>0.05$).

Temperature	Sex ratio (mean ±SE)	Range
23 °C	1.0 ±0.10	0.2 - 1.5
26 °C	2.9 ±0.10	0.8 - 13.3
29 °C	1.0 ±0.20	0.2 - 1.6
32 °C	1.4 ±0.41	1.0 - 2.0
35 °C	1.5 ±0.16	0.8 - 1.8

3.3.2.4 The effect of diet

Trial 1 - The influence of dietary composition on Apocyclops *populations*

Diet exerted a significant influence on *Apocyclops* culture populations. Cultures fed *Heterocapsa*, *Tetraselmis* and a mixture of *Isochrysis*, *Heterocapsa*, *Tetraselmis* and *Rhodomonas* were found to be significantly more productive (mean culture densities equivalent to 2,880 *Apocyclops* L^{-1}) than cultures left unfed or fed either *Isochrysis* or *Rhodomonas* (mean culture densities equivalent to 450 *Apocyclops* L^{-1}) ($p<0.01$, Figure 3.3.13). The highest population density corresponding to 4,870 individuals L^{-1} was recorded from cultures fed a mixed algal species diet corresponding to a cell carbon concentration of 15.2 µgC L^{-1} (Table 3.3.15).

The carbon concentrations (calculated from values in Appendix C5) represented by the cell densities chosen encompassed a wide range of values. The unfed control diet and the *Isochrysis* and *Rhodomonas* diets represented carbon concentrations which were a factor of four times less than that offered in the *Heterocapsa*, *Tetraselmis* and the mixed algal species diets.

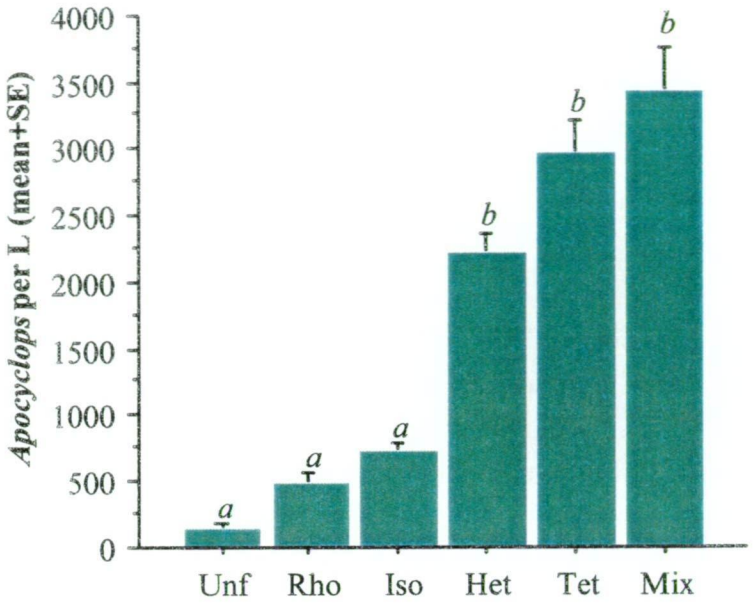


Figure 3.3.13: Influence of diet on *Apocyclops* culture productivity as indicated by the total number of individuals developing in 500 mL over nine days after inoculation with 10 ovigerous females corresponding to an initial density of 5 *Apocyclops* L^{-1}. Italicised superscripts indicate significantly different means as identified by Kruskal-Wallis *k*-sample test and Tukey'smultiple means comparison. Unf – unfed control, Rho – *Rhodomonas*, Iso – *Isochrysis*, Het – *Heterocapsa*, Tet – *Tetraselmis*, and Mix – a mixture of all four algae.

Table 3.3.15: Algal cell carbon density corresponding to the six algal diets offered to *Apocyclops* in the algal species diet trial and subsequent cell carbon density ratio listed in order of increasing *Apocyclops* culture density (Figure 3.3.13).

Diet	Iso µgC L^{-1}	Het µgC L^{-1}	Rho µgC L^{-1}	Tet µgC L^{-1}	Total µgC L^{-1}	Ratio
Unfed	0	0	0	0	0.0	0.0
Rhodomonas	0	0	7.9	0	7.9	2.1
Isochrysis	3.7	0	0	0	3.7	1.0
Heterocapsa	0	24.7	0	0	24.7	6.6
Tetraselmis	0	0	0	24.5	24.5	6.5
Mixed	0.9	6.2	2.0	6.1	15.2	4.1

The influence of diet was evident in the number of nauplii (NI-NVI), copepodids (CI-CVI) and ovigerous females present after nine days of culture. The greater proportion of the *Apocyclops* population (~60%) was represented by nauplii in those yielding greatest overall numbers (Figure 3.3.14a) with a density of 1,865 nauplii L^{-1} present in cultures fed either *Heterocapsa*, *Tetraselmis* or the algal mixture, compared with 17 nauplii L^{-1} corresponding to ~4% of the population in unfed cultures and those maintained on *Rhodomonas* or *Isochrysis*.

Similarly, those cultures fed *Heterocapsa*, *Tetraselmis* and mixed algal species diets also exhibited significantly greater numbers of ovigerous *Apocyclops* (Figure 3.3.14c) representing 40% of the total number of copepodids compared with 0.3% of the copepodids present in the unfed, and *Rhodomonas* or *Isochrysis* fed cultures. The number of copepodids recorded after nine days followed a different pattern (Figure 3.3.14b).

However what appeared to be elevated numbers of copepodids in the unfed, *Rhodomonas*-fed and *Isochrysis*-fed cultures corresponded to ~96% of the population. In contrast, copepodids in *Heterocapsa*-fed, *Tetraselmis*-fed and cultures fed a mixture of the four algae accounted for only ~40% of the population.

Net rates of reproduction indicate *Apocyclops* cultures fed on *Tetraselmis* and the mixed algal diet were the most successful (R_0, 35.3 ±1.8), with *Rhodomonas, Heterocapsa* and *Isochrysis* all exhibiting similar rates (R_0, 17.9 ±1.2) differing from the unfed cultures which yielded a net rate of reproduction value of 2.0 ±0.8 (Figure 3.3.15).

No significant differences were observed between sex ratios for any of the cultures reared on the different diets. The mean sex ratio (female CVI: male CVI) was 4.8 ±0.4.

The day after inoculation, the pH, temperature and salinity levels, recorded for each of the *Apocyclops* cultures fed the six different diets, were not significantly different. *Heterocapsa*-fed cultures exhibited significantly lower dissolved oxygen levels (range of 0.9 to 2.3 mgO_2 L^{-1} corresponding to 12 to 31 percent saturation) than all the other cultures. The mean values for each of the parameters measured are listed in Table 3.3.16 including the data from *Apocyclops* cultures fed *Heterocapsa*.

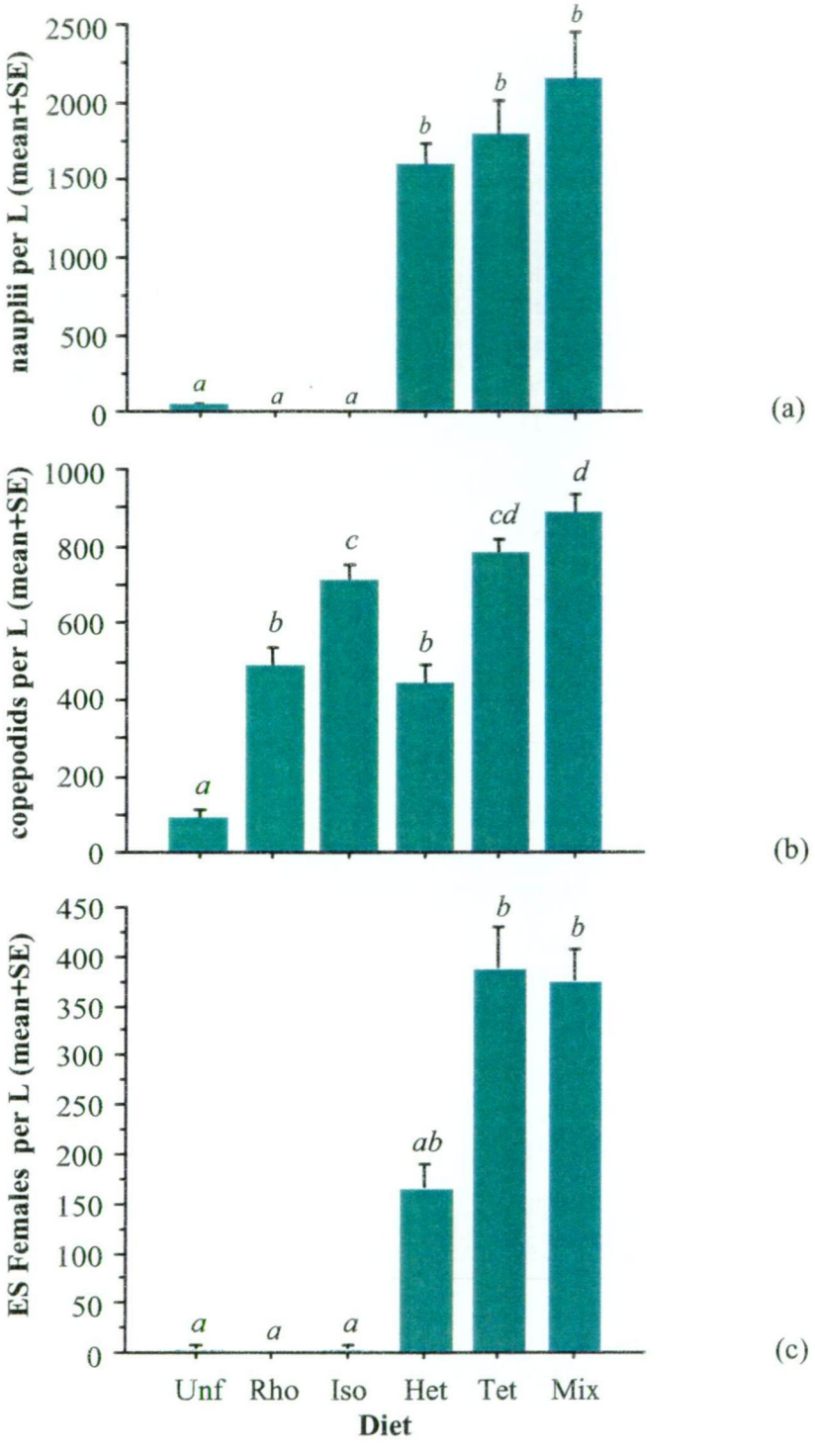
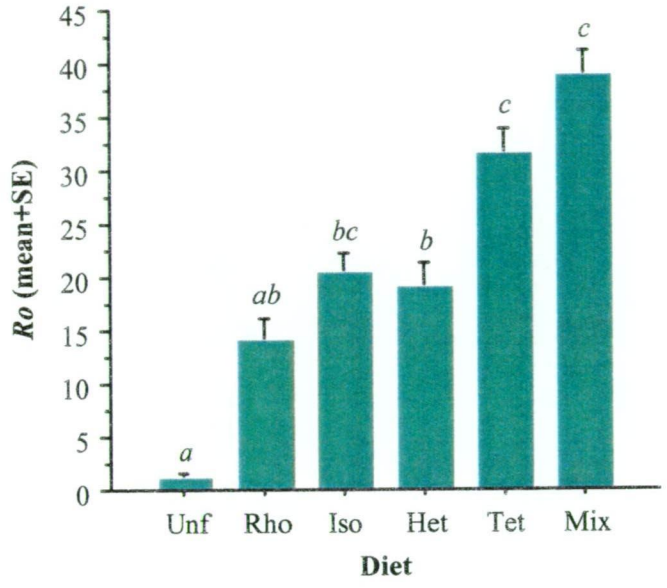
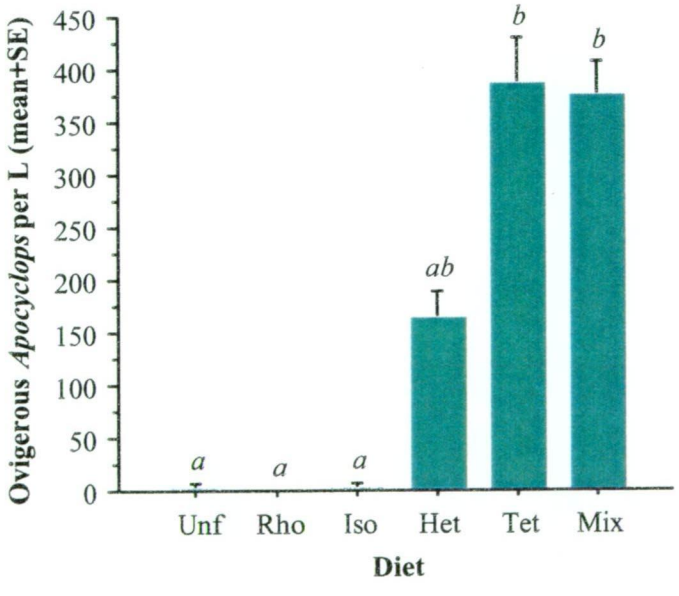


Figure 3.3.14: Influence of algal diet composition on *Apocyclops* culture productivity as indicated by the number of (a) nauplii (NI-NVI), (b) copepodids (CI-CVI), and (c) ovigerous (ES) females present in 500 mL cultures after nine days. Italicised superscripts indicate significantly different means as identified by analyses of variances of data and Scheffe's multiple means comparison. Unf – unfed seawater, Rho – *Rhodomonas*, Iso – *Isochrysis*, Het – *Heterocapsa*, Tet – *Tetraselmis*, and Mix – a mixture of all four algae.



(a)



(b)

Figure 3.3.15: Influence of diet on *Apocyclops* culture productivity as indicated by (a) the net rate of reproduction (R_0) and (b) the number of ovigerous females present after nine days. Italicised superscripts indicate significantly different means as identified by Kruskal-Wallis *k*-sample and Tukey's multiple means comparison. Unf – unfed seawater, Rho – *Rhodomonas*, Iso – *Isochrysis*, Het – *Heterocapsa*, Tet – *Tetraselmis*, and Mix – a mixture of all four algae.

Table 3.3.16: Water quality parameters for all *Apocyclops* cultures one day after inoculation. Measurement means are presented with standard errors (SE) and minimum and maximum values recorded (n=36).

Parameter	Mean	SE	Minimum	Maximum
Temperature (°C)	29.6	0.19	29.6	29.7
pH	8.3	0.03	8.0	8.4
Salinity (‰)	25	0	25	25
Percent saturation (%)	77	6.2	12.2	93.8
Dissolved oxygen (mgO_2 mL^{-1})	5.9	0.48	0.9	7.1

After nine days, the environmental conditions prevailing in the *Apocyclops* cultures fed the six diets differed significantly in terms of pH and dissolved oxygen levels, but they were similar in terms of temperature, salinity and light levels.

The average temperature over the duration of the trial was 29.2 ±0.19 °C ranging from 28.8 to 29.6 °C. Salinity remained constant at 25 ‰, with light levels across the cultures averaging 46.3 ±8.7 lux, ranging from 27 to 61 lux.

In terms of pH and dissolved oxygen levels, the cultures could be divided into two groups. The first (comprising the unfed, *Rhodomonas* and *Isochrysis* fed cultures) exhibited a mean pH of 8.1 ±0.04 with a range from 8.06 to 8.19 associated with a dissolved oxygen values of 1.71 ±0.24 mgO_2 L^{-1}, range 0.14 to 4.5 mgO_2 L^{-1}, corresponding to a mean percentage saturation of 23.1 ±11.6%. The second group (comprising *Tetraselmis, Heterocapsa* and mixed algal species fed *Apocyclops* cultures) possessed a mean 0.5 pH units lower than the first group of 7.6 ±0.06, and range of 7.5 to 7.71. The second group of cultures exhibited dissolved oxygen levels three times higher than the first group at 4.45 ±0.86 mgO_2 L^{-1}, range 3.2 to 5.8 mgO_2 L^{-1}, corresponding to a mean percentage saturation of 65.6 ±13.9%. It is interesting to note that high population densities of *Apocyclops* were present at low levels of dissolved oxygen irrespective of experimental treatment.

Trial 2 - The influence of dietary concentration on Apocyclops populations

Apocyclops population density appears to be directly proportional to algal cell density over the range of $5x10^3$ cells mL^{-1} to $4.6x10^5$ cells mL^{-1} (Figure 3.3.16). The maximum population density recorded was equivalent to 1,985 *Apocyclops* L^{-1} at the highest algal density of $4.6x10^5$ cells mL^{-1} corresponding to a cell carbon concentration of 137 µg L^{-1} (Table 3.3.17).

The greater productivity of the cultures fed higher concentrations of algae is attributable to the greater number of nauplii developing over the nine days (Figure

3.3.17). The cultures reared at algal cell densities greater than $1x10^5$ cells mL^{-1} yielded ten times more nauplii equivalent to 398 ±83 nauplii L^{-1} compared to 20 ±5 nauplii L^{-1} reared at cell densities less than $1x10^5$ cells mL^{-1}. On average, nauplii comprised 38% of the total *Apocyclops* population in cultures reared on diets with greater than $1x10^5$ cells mL^{-1} compared to 5% of the population for cultures reared at lower algal cell densities.

Table 3.3.17: Algal cell carbon density corresponding to the six algal diets offered to *Apocyclops* in the algal cell density diet trial (Trial 2).

Cell Density	*Isochrysis* µgC	*Rhodomonas* µgC	*Tetraselmis* µgC	Total µgC
$1.0 x10^0$	0.00	0.00	0.00	0.00
$5.0 x10^3$	0.07	0.07	0.22	0.35
$1.0 x10^4$	0.13	0.14	0.43	0.70
$1.6 x10^5$	2.13	2.24	6.88	11.3
$3.1 x10^5$	4.13	4.34	13.3	21.8
$4.6 x10^5$	26.0	27.3	83.9	137.2

Variation in the number of copepodids between the two groups of cultures was not as marked. *Apocyclops* cultures maintained at the lower concentration yielded an average 418 ±56 copepodids L^{-1} corresponding to 95% of the culture population, compared to 751 ±129 copepodids L^{-1} corresponding to 73% of the population for those cultures fed algae at concentrations greater than $1x10^5$ cells mL^{-1}.

No significant difference was observed between net rate of reproduction (R_0) calculated for cultures maintained at each of the algal concentrations, with all *Apocyclops* cultures yielded a mean R_0 of 44.6 ±8.1, with a range of 22.8 to 87.6. The average number of ovigerous *Apocyclops* recorded was 221 ±74, with a range of 80 to 666. The higher counts were recorded from cultures fed in excess of $1x10^5$ cells mL^{-1}. The net rates of reproduction observed at the higher cell concentrations were comparable to that obtained on a diet of *Tetraselmis* during the previous trial.

Sex ratio (female CVI: male CVI) was influenced by algal cell density; the higher the algal cell concentration, the lower the sex ratio (Table 3.3.18). The highest average ratio recorded was 9.5 ±0.62 for the unfed cultures, with *Apocyclops* cultures maintained on the highest cell concentration yielding a ratio of 1.4 ±0.21.

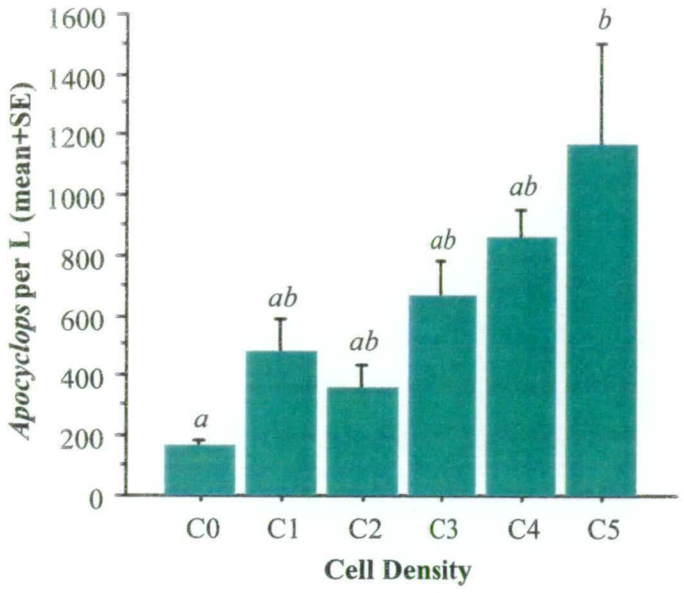
Temperature, salinity and light remained constant across the diets offered. *Apocyclops* cultures experienced a mean temperature of 30.4 ±0.02 °C, salinity of 25 ‰ and mean light level of 45 ±1 lux.

Dissolved oxygen and pH levels showed some variation. Dissolved oxygen, as percent saturation, varied considerably. *Apocyclops* cultures fed the higher algal densities recorded significantly lower oxygen levels, 40 ±4.5% compared with 62 ±4.4%. Conversely, pH varied within 0.3 of a pH unit with no correlation recognised between dissolved oxygen levels and pH. The mean pH level was 8.3 ±0.02, with a pH range from 8.15 to 8.45 recorded, the greatest variation recorded for cultures fed middle level cell densities.

The pH levels recorded in this trial were higher than those observed during the previous trial, ranging from 8.15 to 8.45 (8.3 ±0.2) compared with 7.50 to 7.71 (7.6 ±0.06). The regression analysis conducted with results from the algal species trial revealed a strong relationship between pH and total *Apocyclops* with a coefficient of determination of 81%. The equation generated predicted that minimal productivity would be observed above pH 8.2.

Table 3.3.18: Influence of algal cell concentration on *Apocyclops* population composition after nine days following inoculation with 10 ovigerous *Apocyclops*. R_0 - net rate of reproduction; sex ratio - CVI females: CVI males. Different scripts indicate significantly different means ($p<0.05$) identified by analyses of variances of data and Scheffe's multiple means comparison. C0 corresponds to the unfed control, with C1 to C5 corresponding to the algal cell densities of $5x10^3$, $1x10^4$, $1.6x10^4$, $3.1x10^4$ and $4.6\ x10^5$ cells mL^{-1} respectively.

Algal	Ovigerous		R_0		Sex Ratio	
Density	mean ±SE	$p<0.05$	mean ±SE	$p<0.05$	mean ±SE	$p<0.05$
C0	2.0 ±1.10	*a*	4.8 ±0.52	*a*	9.5 ±0.62	*a*
C1	8.0 ±7.61	*a*	10.4 ±2.80	*ab*	5.7 ±0.73	*ab*
C2	30.4 ±5.30	*ab*	12.8 ±2.73	*ab*	5.7 ±1.17	*ab*
C3	20.7 ±13.75	*ab*	18.8 ±2.91	*b*	3.8 ±0.87	*bc*
C4	119.2 ±8.26	*b*	16.7 ±2.47	*ab*	2.0 ±0.25	*c*
C5	303.6 ±25.4	*b*	25.6 ±6.96	*b*	1.4 ±0.21	*c*



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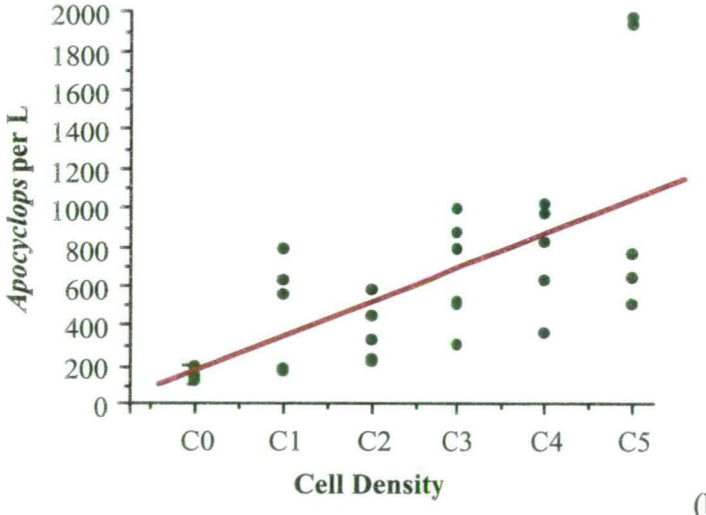
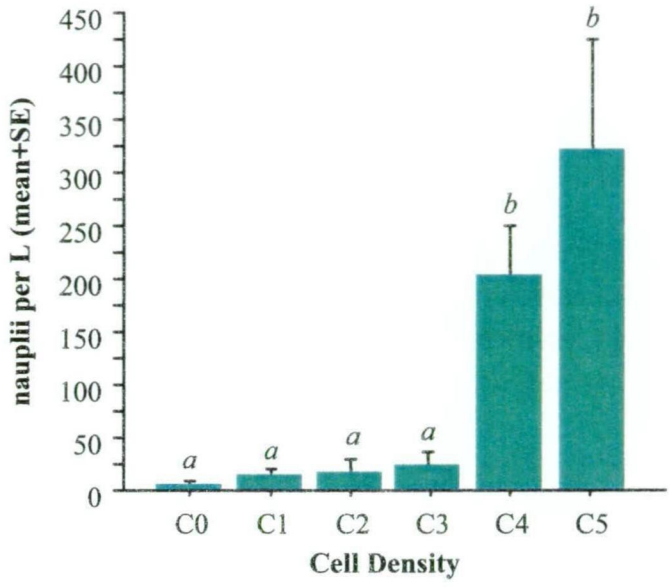


(b)

Figure 3.3.16: Influence of algal cell concentration on *Apocyclops* culture productivity as indicated by the total number of individuals developing over nine days at 30 °C and 25 ‰ after inoculation with 10 ovigerous females. Italicised superscripts indicate significantly different (p<0.05) means as identified by analyses of variances of data and Scheffe's multiple means comparison. The equation of the regression line in (b) associated with a significant level p=0.005 is:

Apocyclops L^{-1} = 172.3 + 174.3*cell density; r^2=0.44.

C0 corresponds to the unfed control, with C1 to C5 corresponding to the algal cell densities of $5x10^3$, $1x10^4$, $16x10^4$, $31x10^4$ and 46 $x10^5$ cells mL^{-1} respectively.



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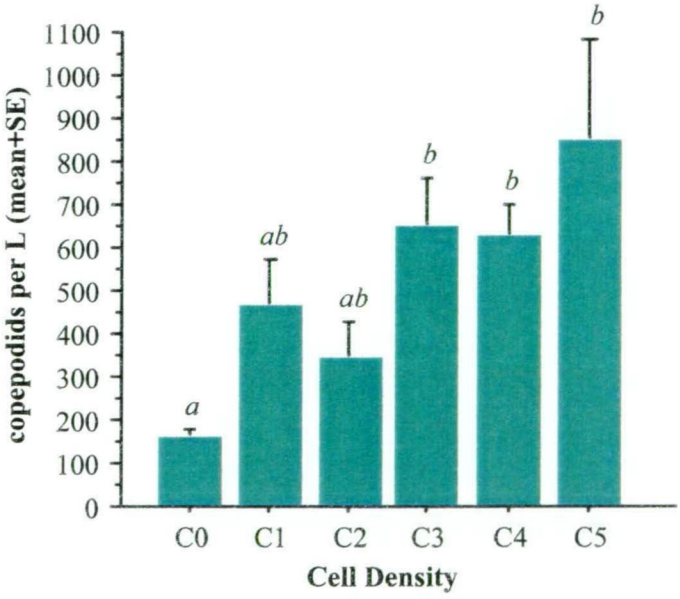


(b)

Figure 3.3.17: Influence of algal cell concentration on *Apocyclops* population composition as indicated by the number of (a) nauplii and (b) copepodids developing over nine days. Italicised superscripts indicate significantly different means as identified by analyses of variances of data and Scheffe's multiple means comparison. C0 corresponds to the unfed control, with C1 to C5 corresponding to the algal cell densities of $5x10^3$, $1x10^4$, $16x10^4$, $31x10^4$ and 46 $x10^5$ cells mL^{-1} respectively.

3.3.3 *Apocyclops*-fish larvae interactions

3.3.3.1 Copepod piscivory as influenced by density and age of barramundi larvae

The presence of *Apocyclops* copepodids did not affect the survival of barramundi larvae at 4, 5, 6 or 7 days post-hatch (dph) ($p>0.05$, Figure 3.3.18). Larvae at 4 dph would appear more susceptible to handling than older larvae as indicated by the significantly lower number of live larvae recovered 24 hours after transfer to aquaria. The decline in numbers is not attributable to copepodid predation as the decline in numbers was also significant in the copepodid-free larval control. It should be noted however that the remains of dead barramundi larvae were still visible in the control aquaria after 23 hours. In contrast there were no remains visible in the treatments containing copepodids, indicating that the *Apocyclops* copepodids do actually consume moribund or dead barramundi larvae.

Significantly, the absence of a decline in barramundi larvae numbers at any of the larval densities or ages investigated does not support the theory that *Apocyclops* copepodids actively predate barramundi larvae.

In contrast, examination of the *Apocyclops* copepodid densities after 24 hours exposure to barramundi larvae revealed that barramundi larvae actively prey upon *Apocyclops* from the age of 6 dph onwards (Figure 3.3.19). There is no significant reduction in the number of *Apocyclops* copepodids by 4 and 5 dph barramundi larvae. However, 6 dph larvae significantly ($p<0.01$) reduced the number of copepodids by up to 50%, with 7 dph larvae possibly consuming 100% of the copepodids inoculated.

Handling effects on *Apocyclops* copepodids appear to be insignificant as determined by the consistent recovery of the same number of *Apocyclops* from copepod control aquaria as were inoculated (Figure 3.3.19).

The temperature range over the duration of the investigation was 27.5 to 29.5 °C with salinity constant at 30 ‰.

3.3.3.2 Copepod piscivory as influenced by health and age of barramundi larvae

Health of barramundi larvae is a significant factor in determining larval susceptibility to predation by ovigerous *Apocyclops*. Healthy barramundi larvae (corresponding to fed larvae of 1 to 8 dph) were unaffected by the presence of *Apocyclops*. In contrast, barramundi larvae starved for between two and five days exhibited reduced survival, both as a result of handling and the presence of *Apocyclops* females (Figure 3.3.20).

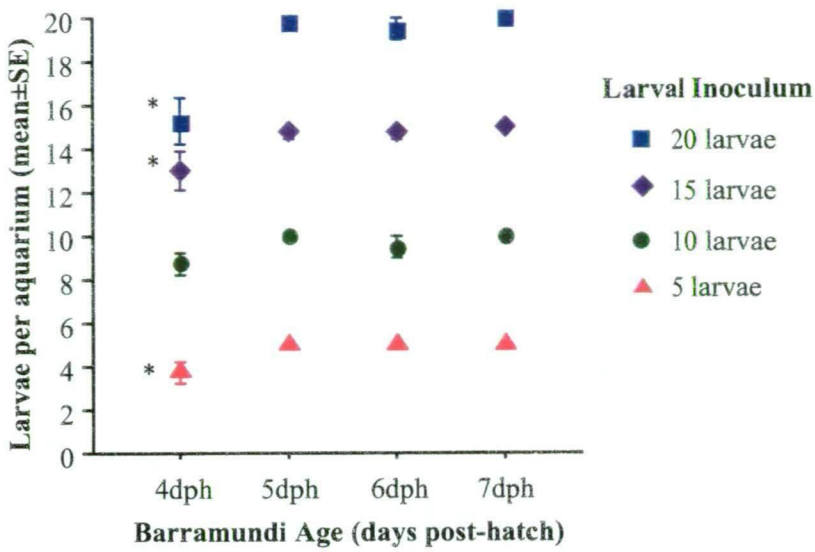


Figure 3.3.18: A summary of the final number of barramundi larvae (mean ±standard error) present after 24 hours exposure to *Apocyclops*. * indicates significantly lower densities at 4 days post-hatch (dph) when compared with older larvae. Where standard error (SE) bars are not visible the magnitude of the SE lies within the dimensions of the symbol.

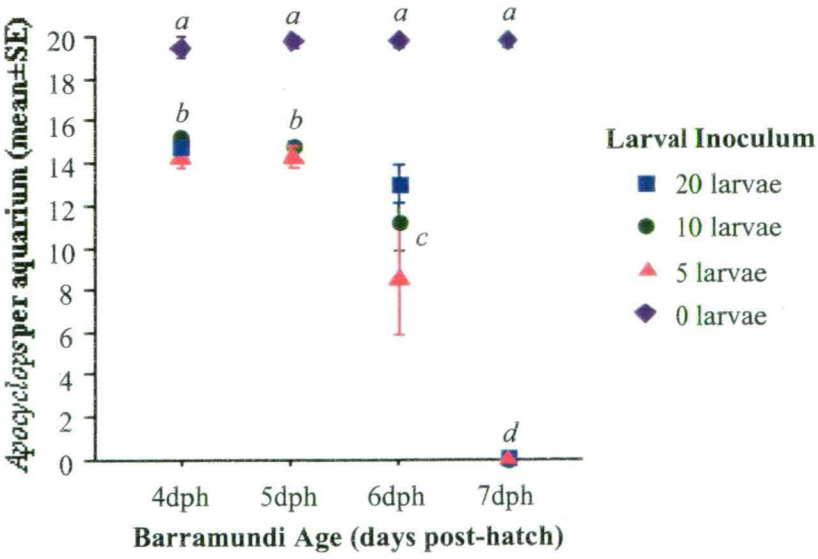


Figure 3.3.19: Influence of larval density on the number of *Apocyclops* (mean ±standard error) remaining after 24 hours exposure to barramundi larval of 4, 5, 6 and 7 days post-hatch. Italicised superscripts indicate significant differences ($p<0.001$) in *Apocyclops* densities as identified by Kruskal-Wallis *k*-sample and Tukey's multiple means comparison tests and correspond to each group of points. Where standard error (SE) bars are not visible the magnitude of the SE lies within the dimensions of the symbol.

The effect of handling and the presence of ovigerous *Apocyclops* had no effect on the survival of 1 and 2 dph barramundi larvae. Handling effects were deemed non-existent as the same numbers of barramundi were recovered after 23 hours as were inoculated. Similarly, the influence of *Apocyclops* was deemed insignificant as the same number of larvae were recovered from aquaria containing barramundi only as from aquaria containing both barramundi and *Apocyclops*.

After the commencement of exogenous feeding, the effect of handling on fed larvae was not significant ($p<0.05$), however unfed larvae suffered significantly from stresses associated with handling at ages 4, 5, 6 and 7 dph (Figure 3.3.20). The numbers of starved larvae recovered from aquaria containing only starved-barramundi being significantly less than the number of fed-larvae recovered from aquaria inoculated with only fed-barramundi ($p<0.05$).

There was no evidence of cyclopoid predation on healthy barramundi larvae of any age investigated in the aquaria containing fed-barramundi larvae and ovigerous *Apocyclops*. In contrast, the number of starved-barramundi larvae recovered after 23 hours from aquaria containing *Apocyclops* were significantly lower from 4 dph onwards. Similarly, the number of starved-barramundi recovered from aquaria containing *Apocyclops* was significantly less than the number of fed-barramundi recovered from 4 dph onwards.

The health of barramundi larvae appears to be more important to survival that the presence of *Apocyclops*. The number of starved barramundi at 4 and 6 dph recovered from copepod-free replicates were significantly ($p<0.05$) more numerous by 1.3 and 0.7 larvae respectively. In contrast, at 5 and 7 dph significantly more ($p<0.05$) starved barramundi were recovered from aquaria containing *Apocyclops* by 3.0 and 1.7 larvae respectively.

The effect of handling on ovigerous *Apocyclops* was not significant in any of the inoculations, nor was the presence of starved barramundi larvae. In contrast, fed larvae at 5, 6, 7 and 8 dph significantly reduced copepodid densities ($p<0.05$; Figure 3.3.21), indicating healthy barramundi larvae may have commenced feeding on ovigerous *Apocyclops* of 1160 µm in length corresponding to a cephalosome width of 370 µm at 5 dph. No examination of larval gut contents was made as the exposure time far exceeded the time for larval digestion to take place.

The temperature range over the eight-day trial was 28.0 to 29.5°C with salinities constant at 30 ‰.

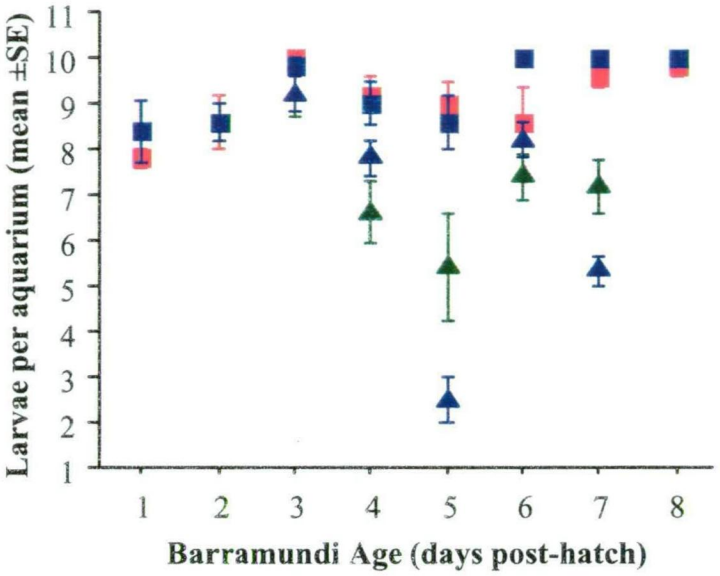


Figure 3.3.20: Influence of barramundi age and health on larval survival after 23 hours exposure to ovigerous *Apocyclops* when initially stocked with ten larvae.

■ fed larvae control

■ *Apocyclops* plus fed larvae

▲ starved larvae control

▲ *Apocyclops* plus starved larvae

Fed barramundi larvae (■ and ■) were unaffected by the presence of *Apocyclops* females across the eight ages ($p>0.05$). Treatments including starved barramundi larvae exhibited significant differences ($p<0.05$) as detected by Kruskal-Wallis *k*-sample and Tukey's multiple means comparison tests at 4, 5, 6 and 7 dph; identifiable where the standard error (SE) bars associated with the ▲ and ▲ do not intersect nearby symbols. Where SE bars are not visible, the magnitude of the SE falls within the dimensions of the symbol.

Starved larvae treatments were not present at 1 dph and 2 dph as a consequence of larvae not commencing feeding until 2 dph. All starved larval stock had died by 7 dph there no starved 8 dph larvae were available.

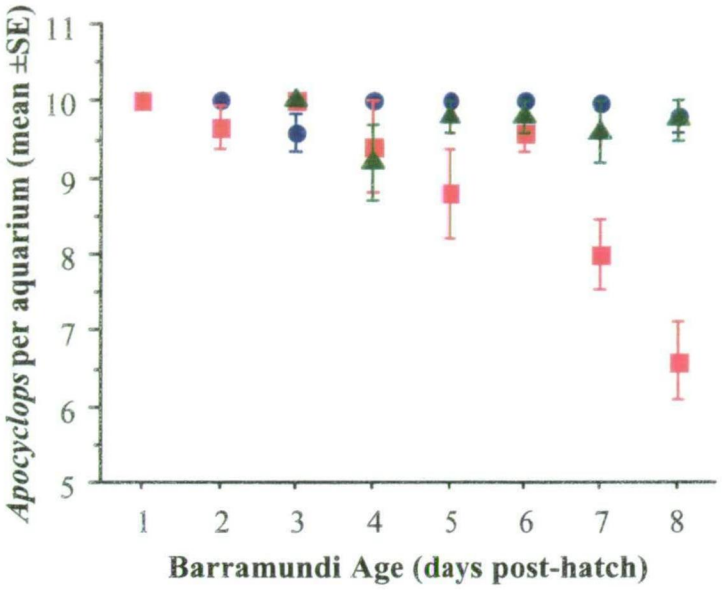


Figure 3.3.21: The number of *Apocyclops* (mean ±standard error) retrieved after 23 hours exposure to barramundi larvae of eight ages: 1, 2, 3, 4, 5, 6, 7 and 8 days post-hatch (dph) when intially stocked with ten copepods.

● *Apocyclops* control, no barramundi larvae

▲ *Apocyclops* plus starved barramundi larvae

■ *Apocyclops* plus fed barramundi larvae

No significant differences were identified in *Apocyclops* numbers between ages for treatments in which no barramundi were included ● or where starved barramundi larvae were present ▲. Significant differences were detected by Kruskal-Wallis *k*-sample and Tukey's multiple means comparison tests where treatments included fed barramundi larvae, identifiable where the standard error bars associated with the corresponding symbol ■ do not intersect nearby symbols.

Where standard error (SE) bars are not visible, the magnitude of the SE falls within the dimensions of the symbol. No starved larvae treatment was present at 1 dph and 2 dph as a result of larvae not commencing feeding until 2 dph.

3.4 Discussion

This study has shown that the Northern Territory species of *Apocyclops* conforms to the general free-living copepod pattern, that is a typical life cycle progressing through twelve readily distinguishable stages from egg through to ovigerous female over 5 to 6 days when maintained at 30 °C. This cyclopoid is able to reproduce over a wide range of temperatures and salinities, on diets of different microalgal species at various concentrations. However, maintenance of *Apocyclops* populations at optimal temperature, salinity and food regimes results in more consistent and slightly higher population densities between culture populations.

From an aquaculture perspective, this copepod meets the majority of criteria for the selection of a larval diet as identified in the general introduction (Section 1.2) presenting life stages of: a) appropriate size, b) being visible and attractive, c) palatable, d) digestible, e) occupying the same habitat as the larvae, and f) exhibiting no obvious piscivorous habit.

This investigation constitutes the fourth published record of *Apocyclops dengizicus* in Australian waters; the first from Lake Buchanan in Queensland in 1965 (Kiefer, 1967) confirmed by Timms (1987), the second from salt lakes on the Eyre Peninsula in South Australia Williams (1984), and the third from saline lakes in northern New South Wales (Timms, 1993). *Apocyclops dengizicus* has also been reported from the tropical and temperate regions of Asia, Africa, South America, North America and throughout Europe (Valderhaug & Kewalramani, 1979; Mirabdullayev & Stuge, 1998).

3.4.1 Life cycle and demographics

Observation of the Northern Territory isolate of *Apocyclops* confirmed the existence of six nauplius and six copepodid stages in the life cycle as reported for the Northern Hemisphere representatives. The north Australian isolate also exhibited similar size increments between successive life stages as documented by Valderhaug & Kewalramani (1979) (Figure 3.4.1). The dimensions of the mature Northern Territory CVI copepodids conform to the figures reported by Mirabdullayev & Stuge (1998).

Length frequency data published by Chang & Lei (1993) for *Apocyclops royi* from tropical Taiwan are similar to those observed for *Apocyclops dengizicus* (i.e. nauplii 110 to 265 µm in length and copepodids 424 to 1034 µm in total length). Male *A. royi* reported to average 0.87 mm in length were 16% smaller than females which averaged 1.03 mm. The 27% difference between genders of the Northern Territory *Apocyclops* may be attributed to the larger size attained by females in Australia.

Distinguishing characteristics for each of the twelve *Apocyclops dengizicus* life cycle stages can be based on length in combination with readily identified anatomical differences as described by Valderhaug & Kewalramani (1979) (Table 3.4.1). Sexual dimorphism is obvious in *Apocyclops* from the fifth copepodid stage onwards. Female *Apocyclops* are larger than males, and exhibit distinctly different antennule morphology. Both male antennules are geniculate (Davis, 1984; Huys & Boxshall, 1991).

The Northern Territory representative of *Apocyclops* exhibits potential as a live food in terms of food particle size. As previously stated the upper particle size limit able to be ingested by marine finfish larvae at the onset of feeding has been reported as 100 µm (Nellen, 1985; Watanabe & Kiron, 1994; Iglesias et al., 1994), and general consensus suggests that prey width is the most important dimensional consideration for ingestion (Shirota, 1970; Ghan & Sprules, 1993; Fernández-Diaz et al., 1994). *Apocyclops* possesses one nauplius stage which meets these criteria: NI possessing a mean width of 91 µm with a corresponding length of 114 µm.

The mean generation time (*T*) observed for *Apocyclops* when maintained at 30 °C and 30 ‰ of 5.3 days was significantly shorter than the 7 to 8 days reported by Valderhaug & Kewalramani (1979) when *A. dengizicus* was reared on *Dunaliella primolecta* at 25 to 28 °C. Dexter (1993) observed *T* ranging from 2 weeks to 2 months at 23 to 25 °C depending on the culture salinity between 0.5 ‰ and 68 ‰. Chang & Lei (1993) reported a *T* of 16 days for *Apocyclops royi* when maintained at 25 °C and 30 ‰. The nauplii of *A. royi* took 0.5 to 1 day to progress through successive stages, a rate comparable to *Apocyclops*. In contrast *A. royi* copepodid stages required 2 days between each ecdysis compared to the approximate 9 hours observed for *Apocyclops*. The differences in *T* evident between the populations may in part be explained by responses to different temperature (Uye, 1988; Klein Breteler, 1994; Takahashi & Ohno, 1996) and diet regimes (Provasoli et al., 1970; Milou & Moraïtou-Apostolopoulou, 1991b; Kleppel & Burkart, 1995). Some of the disparity may be due in turn to geographically-specific characteristics of the cyclopoid populations. Studies conducted by James & Al-Khars (1984) with *A. borneoensis* reared on baker's yeast at 28 °C and 20 ‰ observed a mean generation time of seven days similar to that observed for the Northern Territory *Apocyclops*.

The stage duration and overall mean generation time are important considerations in the provision of live food for larval rearing of fish species. The information is essential to enable the co-ordination of peak nauplius production with the commencement of exogenous larval feeding.

Table 3.4.1: Characteristics differentiating life stages of Australian *Apocyclops dengizicus* combining approximate length data with detailed descriptions modified from Valderhaug & Kewalramani (1979) as provided for the Northern Hemisphere *A. dengizicus*.

Life Stage	Length (µm)	Identifying Features:
N I	115	▪ dorso-ventrally flattened pyriform ▪ posterior margin rounded with two setae ▪ anus absent
N II	170	▪ posterior margin slightly elongated ▪ shallow caudal notch between the two setae
N III	190	▪ posterior margin further elongated ▪ two setae either side of caudal notch
N IV	250	▪ cephalothorax broader posteriorly and narrower anteriorly ▪ caudal notch more defined ▪ maxillule buds evident on ventral surface
N V	280	▪ posterior more elongate ▪ caudal notch more defined with three setae ▪ maxillule segmented
N VI	335	▪ cephalothorax distinctly broad at posterior and narrow anteriorly ▪ caudal lobes bearing 3 setae
C I	445	▪ resembles adult form with broader cephalosome and a narrower urosome terminating in caudal furca ▪ urosome comprises single somite ▪ 2 pairs periopods on first two thoracic somites
C II	575	▪ urosome with 2 somites ▪ 3rd pair periopods appear on 3rd thoracic somites
C III	665	▪ urosome with 3 somites ▪ 4th pair periopods appear on last thoracic somite
C IV	770	▪ urosome with 4 somites ▪ setae represent 5th periopod on 1st urosomal somite ▪ antennule 9 segmented
C V	890	▪ urosome with 4 somites ▪ 5th periopod pair small protuberances ▪ antennule 10 segmented
C VI M	915	▪ urosome with 5 segments 6th periopod represented by setae on 2nd urosomal segment ▪ antennule 11 segmented ▪ both antennules digeniculate
C VI F	1160	▪ urosome with 5 segments ▪ 6th periopod setae absent ▪ antennule 11 segmented, non-geniculate ▪ two egg sacs supported by 2nd urosomal somite

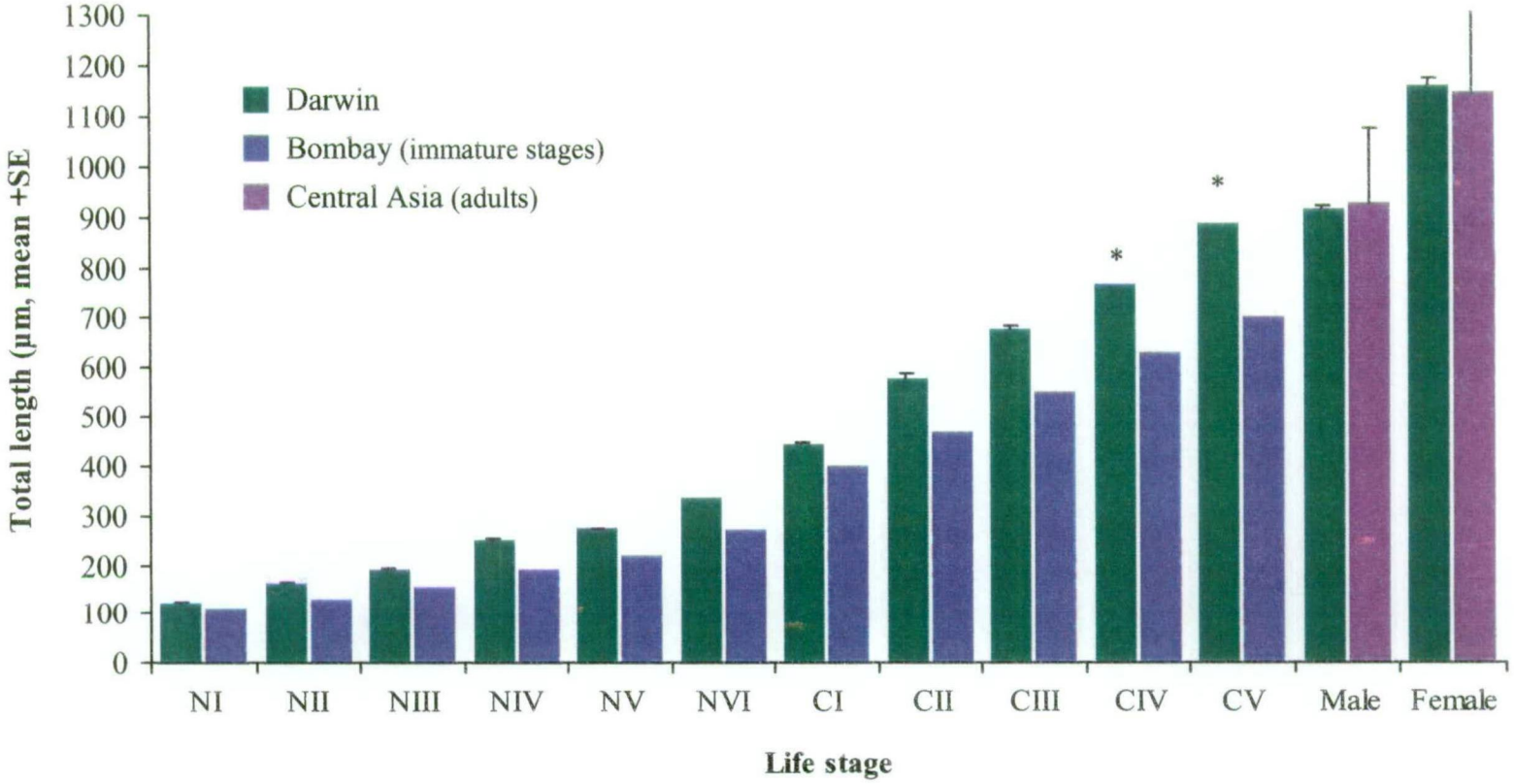


Figure 3.4.1: Comparison of the total length (measured in µm from the rostrum to the end of the last urosomal segment excluding the feruncular setae; mean + standard error) of the life stages of the Darwin geographic type of *Apocyclops dengizicus* with those originating from Bombay (Velderhaug & Kewalramani, 1979) and a combined sample from Kazakhstan and Uzbekistan corresponding to Central Asia (Mirabdullayev & Stuge, 1998). The six nauplius stages are denoted NI through NVI, the sexually immature copepodid stages CI through CV. Sexual dimorphism is evident at stage CV as indicated by the suffices m and f, with sexually mature CVI recorded as Male and Female. * indicates the use of estimated values.

The reported average nine-hour duration of *Apocyclops* life stages and the subsequent 24-hour interval between the appearance of CVI females and ovigerous *Apocyclops* may over-estimate the actual intervals. Chang & Lei (1993) reported a progressive increase in the residence time for each copepod life stage: NI present for a mere few minutes, NII through NIV present for half a day each, nauplius stages V and VI present for one day, with each of the six copepodid stages present for 2 days between each ecdysis. The frequency at which observations were made in this study would not have identified the short residence time of the first nauplius stage. Hopcroft & Roff (1996) suggested that hourly observations of tropical species maintained at 28 °C would barely suffice in determining diel patterns in egg production and naupliar development as a result of the rapid rate of development observed and the associated large variation in development time for each developmental stage in tropical systems.

The attainment of greater demographic detail for each developmental stage is beyond the resources of a single individual, however a team of researchers would be able to do it. And furthermore, the detail may not be necessary with tropical fish larvae developing faster than their temperate counterparts and subsequently being able to ingest larger live food items at a younger age.

3.4.2 Culture of *Apocyclops*

The trials conducted with the Northern Territory isolate of *Apocyclops* showed the cyclopoid to be tolerant of a wide range of environmental conditions. Optimal temperature and salinity ranges were identified, with the influences of diet and culture system found to be variable.

The optimal culture temperature for *Apocyclops* lies between 29 °C and 32 °C as identified by the multi-factorial experiment, and supported by the findings of the replicated single factor trial. *Apocyclops* culture density exhibited a parabolic response to temperature with an indication that temperatures higher than 35 °C would be associated with a sharp decline in culture productivity. The ratio of female to male *Apocyclops* of culture populations across all temperatures was approximately 1.6 with the net reproductive rate in the vicinity of 6 females per male for the optimal range in comparison to that of 2 females per male at the more extreme treatment temperatures of 23 and 35 °C.

The inclusion of individual copepod cultures complementary to mass 500 mL cultures in the single factor trial to obtain more precise demographic information revealed that the additional effort required to obtain greater detail from the progeny of individual females did not provide information beyond that yielded by mass cultures. The minimal information obatined from individual cultures does not warrant their inclusion in future trials assessing a large number of treatment combinations.

The choice of time as opposed to copepod development stage as the factor determining trial duration was made on the basis of the objectives of the thesis – the culture of copepods as a live food. One aspect of culture is the optimisation of culture density which is achievable through rapid population turnover. The disparity observed in the developmental rates between temperatures suggests that total numbers is the most suitable indicator of use for comparison between culture conditions. The final population present in culture after a designated period is an appropriate end point for the assessment of overall effect of that set of conditions on *Apocyclops* population growth, especially considering the resources required to determine the finer demographic detail and the quality of the information generated in terms of the production of live food individuals for aquaculture purposes.

The salinity tolerance study showed female *Apocyclops* could tolerate salinity changes of 20 ‰ increments without detachment of their egg sacs. All cultures across the nine salinities expressed a decline in the number of ovigerous *Apocyclops* over the one-week period with no obvious ill effect at the extreme salinities assessed. Dexter (1993) reported tolerance of salinity changes in the order of two and a half times that of the original medium salinity by the population from the Salton Sea. Although not detailed here, previous trials I had conducted indicated freshwater was lethal within minutes, however successful development and reproduction had been observed at 0.5 ‰ in the laboratory (Dexter, 1993) and at 4 ‰ in Australian saline lakes (Timms, 1993).

Apocyclops non-aerated, static cultures maintained at 20 ‰ yielded consistently higher population densities than those of cultures maintained at other salinities within the range 10 ‰ to 50 ‰. The calculated population densities for *Apocyclops* maintained at 20 ‰ after nine days were equivalent to 4,750 individuals L^{-1}. Subsequent investigations designed to limit confounding influences arising from salinity effects on the nutritive value of diet by using a flow-through system resulted in a final total abundance equivalent to 735 individuals L^{-1} recorded for *Apocyclops* cultures maintained in flow-through systems at 20 ‰.

The greater productivity observed in the static cultures compared to the flow-through systems may have been the result of a number of factors. A minor component may be attributed to culture temperature which was 2 °C lower in the flow-through system. Similarly, the lower density in the flow-through cultures may in part be due to the removal of the original ovigerous inoculum females after four days thus removing five days of naupliar production from the final population density. The use of females from aged cultures combined with the nine day preconditioning of *Apocyclops* to treatment salinities as opposed to wild caught inoculum females is likely to be the main reason behind the lower productivity observed in the flow-through trial. The sex ratio observed for the flow-through cultures (3.4 females per male) was higher than that exhibited by the non-aerated

cultures (2.2 females per male) subject to occasional water exchange, indicative of an aging culture population (Lazzarretto, 1994).

Benefit to *Apocyclops* culture afforded as a result of any reduced effect of salinity on algal growth and quality by the use of a flow-through system was not evident. The apparent negative response may possibly be a consequence of the extended culture of the inoculum *Apocyclops*, or it may be a true reflection of the ability of *Apocyclops* to utilise detritus as food.

Despite the discrepancies in *Apocyclops* densities as apparently influenced by culture system and the history of inoculum females, it is apparent that populations originating from both wild populations and those cultured at salinities between 30 ‰ and 38 ‰ for a number of months, identified 20 ‰ as the salinity most suited to the achievement of dense culture populations of *Apocyclops*.

The multi-factorial trial assessing the influences of temperature, diet, aeration and handling identified the larger microalgae *Tetraselmis* as the most conducive to *Apocyclops* productivity, not *Isochrysis* or fish crumble. Similarly in trials addressing the composition of microalgae based diets, mixed microalgal diets incorporating larger cells, and single species diets of larger cell microalgae, such as *Tetraselmis* and *Heterocapsa*, supported denser *Apocyclops* populations. The success of larger microalgae (cell diameter 13 to 20 µm) may be explained by the dominant raptorial feeding mode adopted within the Cyclopoida (Davis, 1984). In all trials conducted where diet varied, the larger the cell size and the more abundant the microalgal component, the more dense the resultant *Apocyclops* population.

The low population densities observed for *Apocyclops* cultures fed *Isochrysis* and *Rhodomonas* as monospecific diets may not be a true reflection of their value as a food item, but may in fact reflect the low carbon density of the diets at the cell density offered. The Northern Territory isolate of *Rhodomonas* used has been shown to have relatively high levels of lipids and in particular 20:5*n*-3 and 22:6*n*-3 (Renaud et al., 1999). The inclusion of the two species in the mixed diet when accounting for one quarter of the total number of cells present resulted in a more productive population of *Apocyclops* as has been recorded for many copepod species (Vijverberg, 1989).

The influence of aeration on *Apocyclops* population density was shown to be negative. The only environmental parameter significantly affected by the presence or absence of aeration was pH. It is unlikely a difference of 0.3 pH units would impact negatively on a copepod tolerant of such a wide range of environmental conditions. However, culture population density was shown to be related to culture media pH levels with cultures at pH 8.2 supporting less dense populations than those at or near pH 7.6. The adverse response of culture productivity observed

may in part be the result of mechanical interference with the feeding or mating behaviour of *Apocyclops*.

The pH levels recorded during the diet algal cell concentration trial were higher ranging from 8.15 to 8.45 (8.3 ±0.2) compared to the range from 7.50 to 7.71 (7.6 ±0.06) observed during the algal species trial. The regression analysis conducted with results from the algal species trial revealed a strong relationship between pH and total *Apocyclops* with a coefficient of determination of 81%. The equation generated predicted that minimal productivity would be observed above pH of 8.2 which would explain the lower population densities observed in the algal cell concentration trials which yielded maximum *Apocyclops* culture densities in the order of 1,600 individuals L^{-1} compared to 3,600 individuals L^{-1}.

The most productive *Apocyclops* populations in terms of total numbers and nauplii production were identified as 20-25 ‰ and 29-32 °C when fed a diet of *Isochrysis* and *Tetraselmis* at a cell density of 1-2x10^4 cells mL^{-1}, cultures achieving densities of 4,500 *Apocyclops* L^{-1}. The more productive culture populations of *Apocyclops* were those which exhibited a lower ratio of females to males in the order of 1.

3.4.3 *Apocyclops*-fish larvae interaction

The hypothesis that *Apocyclops* is responsible for the failure of green water barramundi crops through predation is not supported by my results. It is more probable that the barramundi crops failed due to factors such as poor larval quality or delayed onset of feeding.

The experiments that I conducted have demonstrated that *Apocyclops* copepodids are consumed by barramundi larvae, and under conditions more akin to semi-intensive larval rearing systems, healthy barramundi larvae are not aggravated or preyed upon by *Apocyclops* copepodids. Where male *Apocyclops* were present in the initial trial, no predation was observed, however, the specific impact of male *Apocyclops* on barramundi larvae was not tested due to the untimely closure of the Darwin Aquaculture Centre.

Barramundi from 3 dph reduce the number of early stage copepodids with 5 dph barramundi able to tackle ovigerous *Apocyclops* 370 µm wide corresponding to a length of 1200 µm. *Apocyclops* nauplii fed to barramundi larvae at the commencement of feeding would not metamorphose into copepodids until the age at which barramundi larvae were observed to impact on the number of ovigerous *Apocyclops*.

There exists some evidence to support the idea that compromised, sick or moribund barramundi larvae fall prey to cyclopoids, but there is no evidence to suggest that early copepodids and mature female *Apocyclops* ever attack healthy barramundi larvae.

The point of no return, when 50% mortality occurs even when live food is offered again (Bagarinao, 1986; Koven et al., 1999), for barramundi is coincident with complete yolk sac absorption occurring at approximately 3 dph depending on water temperature (Bagarinao, 1986; Kohno et al., 1994). Health of larvae was observed by the author to be compromised if larvae were not fed within 24 hours of larval mouth opening at 2 dph (i.e. at 3 dph), and subsequently died even when feed of appropriate size and density were presented. The likelihood of extensively reared larvae starving in ponds usually dominated by rotifers (Appendix B) is remote, therefore larval survival should be ensured unless larval stocking is delayed until after the commencement of exogenous feeding.

Garcia & Alejandre (1995) documented the piscivorous nature of the marine cyclopoids *Corycaeus truckicus* and *C. japnonicus* on the Pacific sardine. These cyclopoids targeted the smaller larvae with 90% of all larvae attacked being less than 7.5 mm. Yolk sac and first feeding larvae were reported from laboratory studies as being more prone to attack by copepods. The associated zooplankton community was dominated by copepod taxa, *Acartia* sp. accounting for 48% of the copepod species followed by *Corycaeus* at 28% and *Paracalanus* at 12%. Similarly, where *Mesocyclops edax* attacked larvae of the striped bass the smaller larvae were targeted, however in all instances less than 1% of larvae came under attack (Cooper, 1996).

It is readily obvious that yolk-sac and first feeding larvae are not as agile as older more developed larvae, predatory copepods concentrating on smaller, less robust fish larvae (Smith & Kernehan, 1981; Cooper, 1996). Observation of the differing interactions between *Apocyclops* and starved barramundi larvae and fed barramundi larvae would suggest that healthy larvae are, if necessary, able to avoid predation by copepods, however compromised larvae may well fall prey to copepod predation.

Preliminary investigations conducted by the author in November 1996 regarding the zooplankton composition of green-water ponds at Barramundi Farms NT revealed the percentage of cyclopoid copepods to be <10% (Appendix B). Of the ponds assessed, the highest cyclopoid proportion was 8% of the total copepod population, the mean for the ponds being 4%. The remaining >92% of the copepod component of the zooplankton comprised primarily calanoid species.

The larval rearing ponds in the process of being 'bloomed-up' (i.e. static pond system with fertiliser added to promote an algal bloom and subsequent zooplankton bloom) supported copepod populations of between 460 and 660 individuals L^{-1}, compared to established ponds (operating on flow-through with no fertiliser added) carrying 1 to 50 copepods L^{-1}. A general two-week cycle was observed with copepod nauplii appearing six to ten days prior to the later stage copepodids becoming more dominant in the zooplankton. Consequently it may be possible that

a one-week window exists within which to stock larval fish and avoid aggravation of the barramundi larvae by adult copepods. Introduction of barramundi larvae to coincide with peak naupliar densities allows three days in which first feeding larvae can develop to a sufficient size to actively prey on all *Apocyclops* life stages by the time that the nauplii have progressed to late stage copepodids.

The trials conducted used higher larval densities than those used in extensive systems, and lower than identified copepod densities (Appendix B). To unequivocally disprove the predatory cyclopoid hypothesis, trials would need to be conducted using lower larval densities and higher cyclopoid densities, including the assessment of male *Apocyclops* interactions with barramundi larvae. Such trials were not undertaken due to the relocation of the Darwin Aquaculture Centre

3.4.4 Summary of findings for *Apocyclops*

The north Australian species in the *Apocyclops dengizicus* species complex exhibits potential as a live food for larviculture. In terms of the objectives identified in the introduction:

- *Apocyclops* exhibits a typical cyclopoid life cycle with twelve free swimming stages ranging in size from 114 µm to 1160 µm in length with a range in width from 91 µm to 368 µm.
- *Apocyclops* exhibits potential as a live food in terms of food particle size possessing at least one nauplius stage (NI) with a width of less than 100 µm (Table 3.3.1).
- Initial separation of stages based on significant dimensional differences is able to be further refined using gross morphological features including segmentation and morphology of antennules and urosome (Table 3.4.1).
- *Apocyclops* exhibits a mean generation time in the order of 5 days at 30 °C, naupliar stages being present for three of these.
- Culture populations maintained at 20-25 ‰ and 29-32 °C and fed a diet of *Isochrysis* and *Tetraselmis* at a cell density of 1-2x104 cells mL-1 achieve densities of 4,500 *Apocyclops* L-1.
- Environmental and food conditions exert significant influences on the population as a whole. The closer to optimal the culture conditions, the greater the population density sustained and the more consistent are the numbers of nauplii present in the population.
- *Apocyclops* was shown not to exhibit piscivorous behaviour. Indeed related species have been recognised as possible replacements for *Artemia* in South

East Asia (James & Al-Khars, 1984; Su et al., 1997; Hsu et al., 2001; Liao et al., 2001).

Chapter 4
Acartia species

4.1 Introduction

Members of the Calanoida are the dominant copepod in marine systems and encompass forty families (Huys & Boxshall, 1991). *Acartia*, the only genus in the family Acartiidae, contains 79 described species which are widely distributed and recorded from all oceans (Uye, 1982; Takahashi & Ohno, 1996). Species of *Acartia* are frequently the dominant copepod in coastal and marine zooplankton and as such have been the subject of numerous ecological studies designed to define trophic systems and their chemical and energy budgets more accurately (Parrish & Wilson, 1978; Pagano & Saint-Jean, 1989; Ederington et al., 1995; Calbet & Alcaraz, 1996; Jónasdóttir & Kiørboe, 1996; Saiz et al., 1997).

The taxonomy of the genus *Acartia* is complex and there are several undescribed species existing in Australian waters (Greenwood, 1972; David McKinnon, AIMS, *pers. comm.*). McKinnon et al. (1992) used molecular techniques to confirm their suspicions, based on morphometrics, that three distinct species, not morphotypes, existed in Victoria.

The taxonomic hierarchy for *Acartia* is detailed in Table 4.1.1.

Table 4.1.1: The taxonomic hierarchy for the genus *Acartia* (after Bowman & Abele, 1982; Huys & Boxshall, 1991).

Level	Title	
Subclass	Copepoda	Milne-Edwards, 1840
Superorder	Gymnoplea	Geisbrecht, 1882
Order	Calanoida	Sars, 1903
Superfamily	Centropagoidea	Giesbrecht, 1892
Family	Acartiidae	Sars, 1903

The value of *Acartia* species as a live food for larviculture has been documented by numerous researchers in the culture of halibut and turbot (Støttrup et al., 1986; Van der Meeren, 1991) and, more recently in the tropics, red-spotted grouper (Toledo et al., 1999) red snapper and rabbitfish (Doi et al., 1997 a,b,c). The majority of scientific reports relate to species of *Acartia* in temperate systems (Pagano & Saint-Jean, 1993; McKinnon, 1996; Hopcroft et al., 1998). More recently expansion of mariculture in South East Asian countries and northern Australia has been associated with an increase in the published literature for tropical copepod species including congeners of *Acartia* (Doi et al., 1994b; Ohno & Okamura, 1988; Lokman, 1994; Sunyoto et al., 1995; Schipp et al., 1999).

4.1.1 Background information for *Acartia* species

The culture of calanoid copepods commenced in the 1960's. *Acartia tonsa* was the first species to be cultured in 1966 followed by *A. clausi* in 1967 (Parrish & Wilson, 1978). Barr (1969) reported culturing *A. longiremis* in 150 mL beakers with the weekly addition of approximately 1 mL of the microalgae *Chaetocerous* species to the culture.

Zillioux (1969) described a system for the continuous culture of planktonic copepods. The recirculation system he used contained ciliates to control bacterial populations and algal debris in combination with foam fractonation to limit the accumulation of dissolved organics. *Acartia clausi* and *A. tonsa* were cultured for over a year with frequent harvesting (Zillioux & Lackie, 1970).

Acartia species can be propagated in tanks and ponds with the possibility of their use as a live food in larviculture proposed by Ikeda (1973). Ohno et al. (1990) found the culture dynamics of *A. tsuensis* were suited to the supply of live food for marine finfish larvae and juveniles in Japan, with *A. sinjiensis* identified as the most important species available for larviculture in eastern Thailand (Ohno et al., 1996).

An understanding of calanoid requirements and the development of appropriate techniques have resulted in the marine species, *Acartia tonsa* being continuously cultivated and used as a food organism for herring, turbot and plaice larvae at the Danish institute for Fisheries and Marine Research (Kiørboe et al., 1984; Munk & Kiørboe, 1985 in Støttrup et al., 1986). The culture of *Acartia* species in tropical climes has occurred in fertilised pond systems inoculated with wild collected zooplankton (Singhagraiwan & Doi, 1993; Doi et al., 1994b; Ohno et al., 1996). The use of *Acartia* species in the larviculture of previously difficult to rear tropical snappers and grouper has resulted in increased survival and growth of subsequent larvae (Doi et al., 1994a; Toledo et al., 1999).

Growth and productivity of copepods in temperate seas are mainly governed by temperature and food availability (Klein Breteler & Schogt, 1994; McKinnon, 1996). Temperature has received much attention whereas salinity has received less attention. The assumption has been made that as the majority of species assessed persist in environments not experiencing large salinity fluctuations, the primary influences on trophic dynamics are temperature and food. The level of interest in estuarine and brackish water environments may see a change as indicated by Pagano & Saint-Jean's work (1989, 1993, 1994). The influence of temperature on hatching, growth, and fecundity, among other things, has been well investigated for a number of *Acartia* species (Takahashi & Ohno, 1996). Optimal conditions have been identified for temperate species, however the optimal ranges for tropical species are less well publicised. In general the relationship between development time and temperature can be represented by Bělehràdek's formula (Klein Breteler

& Schogt, 1994; Takahashi & Ohno, 1996) with the rate of development increasing with increasing temperature and food level to an optimum beyond which rates decline.

Calanoids have been established as omnivorous feeders capable of selective predation (Gifford & Dagg, 1988; Vanderploeg, 1994; Ederington et al., 1995). Although Norsker & Støttrup (1994) suggested that calanoids are largely confined to eating microalgae, as they are primarily filter feeders on smaller particles, no calanoid is truly herbivorous with *Acartia* species exhibiting omnivorous tendencies feeding on larger particles such as ciliate protozoans in a raptorial manner (Allan, 1976; Vanderploeg, 1994; Ederington et al., 1995).

Ontogenetic differences exist between nauplii, juvenile and adult copepodids in their ability to select and capture particles (Swadling & Marcus, 1994). Nauplii tend to feed non-selectively, with copepodids exhibiting variable degrees of food selection.

The lower size limit for particle capture determined for *Acartia tonsa* was between 2 µm and 4 µm for all developmental stages (Berggreen et al., 1988). Optimal particle size was found to increase during development corresponding to 2 to 5 % of the prosome length (Berggreen et al., 1988). The optimal particle size for nauplii of ~7 µm was associated with an upper limit of 10 to 14 µm. The optimal particle size for adults ranged from 10 to 70 µm with a corresponding maximum size limit of ~250 µm (Berggreen et al., 1988).

The studies on the productivity of *Acartia* species have focused heavily on egg production as the genus exhibits broadcast spawning and eggs are supposedly relatively easy to collect (for example Støttrup et al., 1986; Sunyoto et al., 1995).

Calbet & Alcaraz (1996) demonstrated that alternating periods of high and low food availability were reflected in egg production by *Acartia grani*. Starvation for 48 hours resulted in the cessation of egg production, with egg laying resuming 24 hours after the start of feeding. The observed recovery time was also influenced by temperature. Females at 23 °C resumed egg production in two thirds the time of females maintained at 18 °C. Similarly, *A. tonsa* was observed to cease egg production entirely after four days upon transfer from a mixed diet to a monospecific diet of *Dunaliella* (Støttrup & Jensen, 1990). The poor performance of *A. tonsa* was attributed to the nil or only trace levels of HUFA present in the microalgae (Støttrup & Jensen, 1990).

In contrast, *Rhodomonas* species have been consistently associated with productive *Acartia* individuals (e.g. Støttrup et al., 1986; Berggreen et al., 1988; Jónasdóttir, 1994), such findings attributed to the fatty acid composition of the microalgae. The amino acid and vitamin content of *Rhodomonas* species may also contribute to success of the microalgae as a diet (Jónasdóttir & Kiørboe, 1996).

A theme under constant review is the potential inhibition by diatoms of copepod egg production and hatching. The phenomenon has been observed with *Acartia*

species fed *Thalassiosira rotula* (Ianora et al., 1996). Ianora et al. (1996) determined that inhibition of egg production was caused by intracellular components, not bacteria associated with the diatoms, as axenic diatoms produced the same level of inhibition as that observed when non-axenic diatoms were used. However Jónasdóttir & Kiørboe (1996) found that negative effects of high concentrations of diatom extract were resolved by aeration, hypoxic conditions in the extract being the cause of hatching failure. The importance of diatoms as a high-quality food for copepods in natural environments may still hold good.

The Northern Territory calanoid species studied here was identified by Dr David McKinnon (Australian Institute of Marine Science, Townsville) as belonging to the *Acartia plumosa* species group within the subgenus *Acanthacartia*. No species name could be assigned because of the taxonomic complexity of the genus. Voucher specimens comprising 10 male and 10 female *Acartia* and the details of their origin have been deposited with the Museum and Art Gallery of the Northern Territory catalogue number NTM Cr012709.

Throughout the remainder of the thesis, unless indicated otherwise, the name *Acartia* relates specifically to the Northern Territory species.

4.1.2 Objectives

The aim of the chapter is to document the culture characteristics of a tropical Australian *Acartia* species and assess its suitability as an alternative life food for marine larviculture using golden snapper as a test species. Three distinct areas were investigated: (a) life cycle, (b) culture and (c) larval feeding trials.

The objectives of the work undertaken were to:

A. Document the number and sizes of each of the stages in the life cycle of *Acartia*.

B. Describe the gross morphological features of each stage so that it is possible to differentiate developmental stages during culture.

C. Determine the mean generation time and duration of each of the life stages of *Acartia*.

D. Identify the influence of salinity, temperature, light level, culture volume, stocking density and diet on the growth and development of *Acartia*.

E. Assess the effects of salinity, temperature and diet composition on culture population density and demographic variables of *Acartia* such as population structure, net rate of reproduction and sex ratio.

F. Assess the performance of golden snapper larvae when presented with *Acartia* in terms of growth and survival of the larvae in small volume cultures.

4.2 Materials and methods

Common methods

Throughout the chapter all water quality parameter measurements were taken using the same equipment and methods as those detailed in the Common Methods of the previous chapter (Section 3.2). Similarly the four microalgae used: *Isochrysis*, *Heterocapsa*, *Rhodomonas* and *Tetraselmis*, are the same species described there and they were maintained under the same culture conditions (Section 3.2). The details of the two additional microalgae presented to *Acartia* are:

a. *Nitzschia palacea* (Northern Territory University collection number NT7) referred to simply as *Nitzschia* in the remainder of the chapter. The Darwin Harbour bascillariophyte exhibits a mean cell size of 17.5 x 2.5 µm.

b. *Cryptomonas* sp. (Northern Territory University collection number CRF101) referred to simply as *Cryptomonas* in the remainder of the chapter. The Darwin Harbour cryptomonad exhibits a mean cell size of 7.4 x 4.2 µm.

4.2.1 Life cycle and demographics

Interest in *Acartia* by the Australian aquaculture industry is relatively recent and this copepod is essentially unknown to the majority of aquaculturists. The objectives of the following investigations were to observe the life cycle *Acartia* and identify key characteristics of importance to aquaculture technicians in the culture and use of it as a live food for marine finfish larviculture.

4.2.1.1 Life cycle

Copepod material was collected at night from Vesty's Lake, a tidal lagoon in Darwin, Northern Territory, Australia (S 12° 25.970' E 130° 50.199'), using light as an attractant and concentration tool, in conjunction with a 150 µm mesh hand scoop net. The copepods collected were passed over a 5 mm mesh to remove *Acetes* shrimp and fish larvae prior to being rinsed with filtered seawater in the 'plankton washer' fitted with a 190 µm mesh screen to remove any rotifers (Section 3.2.1).

The clean *Acartia* copepodids were transferred to a 100 L Nally® bin containing 80 L of fresh culture media at 35 ‰ supporting a light bloom of *Tetraselmis* and *Isochrysis* at approximately 10^4 cells mL^{-1}. A 250 Watt Jäger aquarium heater was added to the culture to prevent the outdoor culture temperature from dropping below 29 °C as the overnight temperatures during the 'dry season' (winter, April through October) frequently drop as low as 18 °C. Aeration supplied via a weighted 4 mm airline delivered air at approximately 2 L $hour^{-1}$ to assist in maintaining an homogenous environment. The culture was maintained outdoors under natural light conditions (photoperiod 11 L:13D) for a period of three and a

half weeks with the regular addition of *Tetraselmis* and *Isochrysis* when the culture media cleared. Temperature, salinity, dissolved oxygen and pH fluctuations were monitored daily, with ammonia and nitrite levels monitored on alternate days.

A representative sample of the *Acartia* population was collected on alternate days using a 70 mL beaker dipped at five points around the 100 L culture unit: four equidistant around the outside of the culture, the fifth taken from DAC. The sample was screened over a 44 µm mesh screen and the retained copepod stages were preserved in 5% formal saline. The *Acartia* population samples were sorted into size classes: small nauplii (NI-III), large nauplii (IV-VI), small copepodids (CI-IV) and large copepodids (CV-VI) under an Olympus SZ40 stereo dissecting microscope. Adult male and adult female copepodids (CVI) were recorded for each sample under high magnification (64x) on an Olympus SZ40 stereo dissecting microscope.

A subsample from each collection was then mounted in 5% formal saline and cover-slipped for size determination. Measurements were made under bright-field conditions using an Olympus BH-2 compound microscope fitted with 4x, 10x, 20x and 40x objective lenses, in combination with 10x eyepiece lenses.

4.2.1.2 Demographics and productivity

Isolation and subsequent culture of individual ovigerous *Acartia* was not possible. A number of attempts to isolate various stages of the calanoid were unsuccessful despite the use of a variety of techniques including anaesthesia using MS222 and cooling of cultures, a variety of culture vessels ranging from 2 mL tissue culture wells through to 250 mL cylindrical plastic containers. Similarly, it was difficult to obtain viable eggs from which to create a distinct artificial cohort (Appendix C2). Consequently in order to obtain estimates of mean generation time and life stage duration, mass cultures were monitored on a regular basis in order to track the most abundant life stage at each time interval.

Life stage abundance data were collected daily from four 10 L cultures. The medium scale cultures were inoculated at 100 *Acartia* copepodids L^{-1} and maintained at 35 ‰ and 29.4 °C on *Rhodomonas* and *Tetraselmis* at $8x10^4$ cells mL^{-1} for sixteen days. Individual cultures were sampled daily to yield a 500 mL volume sample which was preserved with the addition of 50 mL of a 1:1 mixture of formalin and glycerol.

Water quality parameters were monitored twice daily, early morning prior to sampling, and after the cultures were fed 500 mL of the appropriate algal mixture to maintain the algal cell concentrations. The full details of the culture maintenance regime are presented in Appendix C6 Medium Scale Algal Species Trial.

Statistical analyses

Statistical analyses were employed to ascertain the value of life stage dimensions as a diagnostic characteristic as described for *Tisbe* in Section 2.2.1 using material collected from the 80 L *Acartia* cultures.

4.2.2 Culture of *Acartia*

Acartia was observed to be far less tolerant of handling than either *Tisbe* or *Apocyclops*. *Acartia* being a broadcast spawner not carrying external egg sacs translated into a need to develop alternative methods for isolation of inoculum and modification of culture techniques to favour growth of *Acartia* populations.

During the temperature and salinity experiments it was found that light and culture volume played a significant role in *Acartia* productivity. Consequently artificial and natural light levels were assessed in culture volumes from 150 to 500 mL.

Similarly the difficulty experienced in obtaining consistent supplies of *Acartia* life stages with which to inoculate trials prompted an investigation into the minimum stocking density required to produce consistent culture densities within treatments.

A more comprehensive understanding of the influences of temperature, salinity light and culture volume enabled the completion of trials assessing the impact of diet composition on *Acartia* culture population density.

Acartia stock culture

Acartia copepodids were collected from Vesty's Lake and rinsed in clean seawater as described above prior to distribution between two 100 L cultures containing a mixture of *Isochrysis*, *Rhodomonas* and *Tetraselmis*.

The *Acartia* stock cultures were gently aerated by two 4 mm airlines, each fitted with a ceramic weight and 2 L hour^{-1} flow regulator. The cultures were fed a daily ration of the three algae, which lightly coloured the culture media corresponding to a final cell density of approximately $2x10^4$ cells mL^{-1}. The cultures were maintained outside, shaded from direct sunlight (~6000 lux) but exposed to ambient temperature of 28 to 32 °C and photoperiod of 13 L:11D.

Every 8 days the entire culture was rinsed in the 'plankton washer' fitted with a 190 µm-mesh screen to remove ciliates. The copepodids were concentrated and 100% of the culture medium renewed. Joint trials with DAC staff had determined optimal timing to maximise *Acartia* production (see enclosed paper by Schipp, Bosmans & Marshall, 1999 - Appendix D).

The adult *Acartia* used as the inoculum for trials were obtained from the 100 L cultures seven days after screening, at which time the first filial generation was known to dominate the culture population (Schipp et al., 1999). The inoculum was

collected by gently screening a portion of the culture over a submerged 150 µm-mesh screen; *Acartia* copepodids were transferred to the experimental culture units using a 25 mL glass beaker.

4.2.2.1 The effects of light source and culture volume

This trial was designed to address the effects of light and volume taking into account the findings of the preliminary trial indicating a need for culture volumes larger than 150 mL and a requirement for shading of cultures exposed to natural ambient conditions outside (Appendix C3). The aim of this trial was to test the influence of light intensity and volume on the productivity of *Acartia* cultures maintained on a diet of *Tetraselmis* and *Isochrysis*.

Four 100 L water baths were each supplied with a heater and aeration to achieve consistent temperature between light treatments. One water bath was exposed to each of the four light treatments experiencing a 13L:11D photoperiod:

AL - artificial low intensity light (~40 lux)
inside with no additional light other than the background light provided by Osram fluorescent tube ceiling lights.

AH - artificial high intensity light (~500 lux)
inside with an additional incandescent light source operating on a timer.

NL - natural low intensity light (~30,000 lux)
outside floating in a 7 m^3 tank under a canvas shade sail.

NH - natural high intensity light (~60,000 lux)
outside floating in a 7 m^3 tank with no shading.

Twenty-four 150 mL culture units were each inoculated with 18 *Acartia* copepodids and twenty-four 500 mL culture units were inoculated with 60 *Acartia* copepodids, corresponding to a density of 120 *Acartia* L^{-1}. The culture medium in each unit was maintained at a salinity of 35 ‰ and supplemented with *Tetraselmis* at $6x10^4$ cells mL^{-1} and *Isochrysis* at $1x10^5$ cells mL^{-1}. Seven 150 mL and seven 500 mL replicate culture units were placed under each of the four light regimes: six containing *Acartia*, the seventh copepod free.

Light intensity, temperature, salinity, dissolved oxygen and pH levels were monitored daily in the 150 mL and 500 mL culture units containing only algae.

The trial was terminated after five days at which time a 5% solution formalin glycerol solution was added to each culture unit. The number of adult male, adult female, late copepodid (CIV and CV), immature copepodid (CI-CV), late naupliar (NIV-NVI), early naupliar (NI-NIII) and egg stages of *Acartia* present in each sample were determined using an Olympus SZ40 dissecting microscope.

4.2.2.2 The effect of stocking density

Trials conducted with *Acartia* were only able to be inoculated with copepodids. Numerous attempts had been made to achieve standard inoculation with a known inoculum, however aspects of handling meant that the life stages inoculated did not develop, or insufficient numbers of the desired life stage were attainable. The aim of the following trial was to determine the optimum mixed copepodid stage *Acartia* inoculum.

Six replicate culture units were inoculated at each of 7 densities: 0, 10, 20, 40, 80, 160 and 320 *Acartia* L^{-1}. Culture media containing *Tetraselmis, Isochrysis* and *Rhodomonas* at $6x10^4$, $1x10^5$ and $6x10^4$ cells mL^{-1} respectively was made up using axenic algae cultures and 0.1 µm filtered seawater at 35 ‰.

The forty-two culture units consisted of a 500 mL cylindrical container supplied with fresh media daily from an aerated 1 L header tank, the contents of which were gravity fed via a 2 L/hr dripper into the 500 mL container. Excess culture media passed through a 63 µm mesh screened outflow into a receptacle (similar to the system described in Section 2.2.2.5). The entire system was maintained in a constant temperature room at 30 ±0.4 °C with a 13 L:11D photoperiod regime. The results from experiment 4.2.2.1 showed that supplementary lighting by 36W OSRAM 'flora' fluorescent tubes suspended directly above the 500 mL culture units providing a mean light intensity of ~1400 lux (see photograph Figure 4.2.1 Section 4.2.2.4) was required for optimal production.

The *Acartia* cultures were maintained as described for 7 days after which time the entire contents of each 500 mL culture unit was preserved in a 1:1:8 mixture of formalin, glycerol and culture medium with the number of individuals in each life stage group determined as detailed in 4.2.2.1.

4.2.2.3 The effects of salinity and temperature

Temperature and salinity are two aspects of artificial culture which can be readily manipulated and are also recognised to have strong influences on copepod growth and development through their regulation of metabolic functions (Hart, 1990; Takahashi & Ohno, 1996). This trial was undertaken to investigate whether any interaction between temperature and salinity was evident in terms of *Acartia* culture population density after nine days exposure to 35 treatment combinations encompassing the temperature range from 23 °C to 35 °C and the salinity range from 5 ‰ to 45 ‰.

Ten 100 L water baths were setup and calibrated as described in Section 2.2.2 one week prior to the start of the experiment. Duplicate water baths were set to each of the treatment temperatures 23 °C, 26 °C, 29 °C, 32 °C and 35 °C arranged in two rows of five, corresponding to blocks, in a constant temperature room maintained

at 22 ±2 °C. Water bath temperatures were monitored twice daily and adjusted where necessary.

Culture media were made up in batches using a combination of Aquasonic® *Ocean Nature* artificial sea salt, aged tap water and algae to achieve the seven treatment salinities: 5 ‰, 15 ‰, 20 ‰, 25 ‰, 30 ‰, 35 ‰ and 45 ‰, with *Isochrysis* at $1.0x10^5$ cells mL^{-1} and *Rhodomonas* at $6.0x10^4$ cells mL^{-1}. Nitrite and ammonia levels of the fresh media were recorded prior to the distribution of 200 mL volumes to each of the seventy culture units.

Acartia were collected from Vesty's Lake on a night approaching a full moon and rinsed as described previously. The copepodids were introduced into a 40 L culture comprising a lightly aerated mixture of *Rhodomonas* and *Isochrysis* maintained under natural light and temperature conditions for a period of two days.

On day zero (D0) of the trial each culture unit was inoculated with 20 *Acartia* copepodids as trials conducted using eggs and nauplii as the inoculum life stage failed to develop culture populations (Appendix C3).

A 75% culture medium exchange was conducted every third day. The spent culture medium was siphoned out through a 44 µm mesh screen to prevent the removal of any *Acartia* and replaced with freshly prepared, temperature acclimated culture media of the appropriate salinity. The salinity, pH and DO levels of the spent media were recorded, and the dominant copepod life stage present in the culture unit noted.

The trial was terminated after nine days, which was deemed sufficient time for inoculum copepodids to mature and resultant offspring to develop given a mean generation time of 8 days (Section 4.2.1.2). All culture units were siphoned down to 50 mL through the 44 µm mesh screen, and preserved with a 1:1 mix of glycerol and formalin added at 5% with the number of individuals in each life stage group determined as detailed in Section 4.2.2.1. Final temperature and salinity measurements were recorded.

4.2.2.4 The effect of diet

In conjunction with temperature and salinity, diet is the most easily manipulated culture condition, and also one of the most important from the perspective of growth and development of copepods.

Acartia species have been reported as primarily algal feeders (Norsker & Støttrup, 1994; Vanderploeg, 1994). The following trial was designed to assess the importance of diet composition on *Acartia* population density as determined by numbers developing in cultures fed either mono-specific and mixed microalgal species diets.

Feeding and reproduction of *Acartia*, like all other copepods, is governed by the quality and abundance of available food, with size, shape and biochemical composition being important aspects of food quality (Berggreen et al., 1988; Støttrup & Jensen, 1990; Jónasdóttir, 1994; Vanderploeg, 1994; Ederington et al., 1995). The majority of literature reporting diet preferences of *Acartia* are primarily based on temperate species with little work published for tropical species.

The six algal species assessed in the following trials were chosen for a variety of reasons. *Isochrysis* and *Tetraselmis* are two algae commonly used in aquaculture because of their appropriate size range for rotifers, nutritional composition and therapeutic effects on water quality (Kellam & Walker, 1989; Støttrup et al., 1995). No heterotrophic algae or artificial diets were assessed because of the ready supply of microalgae and previous difficulties experienced with artificial enrichment products in tropical environments when rapid putrification of culture media occurred (Schipp, *pers comm.*).

The local *Rhodomonas* isolate was chosen as it formed a natural component of the calanoid diet in the wild in addition to exhibiting an appropriate cell size and literature citing successful rearing of *Acartia* with *Rhodomonas baltica* (Sorgeloos et al., 1988; Støttrup & Jensen, 1990; Klein Breteler et al., 1990).

The dinoflagellate *Heterocapsa* was selected because of its large cell size and ability to remain in suspension with minimal aeration. Adult *Acartia* species are reportedly unable to feed efficiently on algal species smaller than 10-12 µm (Berggreen et al., 1988).

Renuad et al. (1994) also recommended the Northern Territory isolate of *Nitzschia palacea* as a suitable component of a mixed microalgal diet for mariculture organisms in tropical aquaculture due to its lipid and protein content.

The following trials were designed to assess the effects of algal species and algal carbon concentrations on *Acartia* culture productivity and to determine the most appropriate species composition and cell density for mass culture of *Acartia*.

Determination of algal rations

Dr Bob Campbell (Marine Research Scientist, University of Rhode Island) indicated that the concentration of 500 µg carbon L^{-1} was the minimum concentration required for maximum growth when using small flagellates indicating lower concentrations may be more appropriate when larger microalgae were included in mixed diets. Cell carbon levels were estimated by direct measurement of cell dimensions in conjunction with formulae devised by Eppley et al., 1970 (Smayda, 1978). The resultant calculations yielded desired densities of 5.7 $x10^4$ cells mL^{-1} for *Tetraselmis*, 6.8 $x10^4$ cells mL^{-1} *Rhodomonas*, and 2.0 $x10^5$ cells mL^{-1} for *Isochrysis*, 1.4 $x10^5$ cells mL^{-1} for *Cryptomonas* and 1.7 $x10^5$ cells mL^{-1} for *Nitzschia* corresponding to a cell carbon concentration of 500 µg L^{-1}

(details provided in Appendix C5). The values presented in Table 4.2.1 are those on which all ration compositions were based.

Table 4.2.1: Calculated cell volume and carbon concentration values for each of the algal species used in dietary trials with *Acartia* based on the formula of Eppley et al. (1970, IN Smayda, 1978).

Algal species	Estimated Cell Volume (μm^3)	Estimated pgC $cell^{-1}$
Cryptomonas	104	3.6
Isochrysis	104	2.5
Heterocapsa	1776	234
Nitzschia	86	2.9
Rhodomonas	230	7.3
Tetraselmis	129	8.7

Copepod collection and culture

Acartia were collected from Vesty's Lake and rinsed overnight in the 'plankton washer' as described previously. Two 100 L culture vessels supplied with gentle aeration were maintained outside under shade and supplied a mixed diet of *Tetraselmis, Isochrysis, Rhodomonas* and *Heterocapsa* for a total of four days. Late stage copepodids were used as the inoculum with 20 individuals pipetted into each of the 500 mL experimental units using a wide bore (Ø 3 mm) plastic pipette.

The effect of algal species

The performance of *Acartia* populations when fed one of twelve microalgal diets were assessed in terms of total numbers developing during six days of culture. The trial was designed to assess the potential of *Nitzschia* and *Cryptomonas* as diets for *Acartia* when presented alone or as a component of mixed microalgal species diets. Concern was expressed as to the potentially harmful nature of *Nitzschia* being a diatom and related to species proven to inhibit *Acartia* egg development (Ianora et al., 1996) with the production of anti-mitotic compounds possibly ubiquitous amongst diatoms (Uye, 1996). Treatment diets were made up to achieve a final salinity of 35 ‰ identified as the optimal salinity for maximum *Acartia* population growth in Section 3.2.2.3. The algal cell densities used were double those listed in Table 4.2.2 to ensure *Nitzschia* were present in reasonable numbers.

Four replicate culture units were filled with 500 mL of the treatment media for each of the twelve diets. The 48 culture units consisted of a 500 mL cylindrical container supplied with fresh media from an aerated 10 L header tank, the contents of which were gravity fed via a 2 L/hr dripper at a rate of 300 mL hr^{-1}. Excess

media passed through a 63 µm mesh screened overflow. The entire system was maintained in a constant temperature room at 29 °C with a 13 L:11D photoperiod regime. Supplementary lighting was supplied as indicated in Section 4.2.2.2 (Figure 4.2.1).

Temperature, salinity and dissolved oxygen levels were recorded on a daily basis for each culture unit prior to the reservoirs containing the treatment diets being emptied, rinsed and replenished with the respective algal mix. Culture media were maintained at 35 ‰ identified as the optimal salinity for *Acartia* population growth (Section 4.3.2.3).

The trial was terminated after six days at which time final temperature, salinity, pH, dissolved oxygen and incident light level measurements were taken prior to the entire contents of each unit being passed over a 44 µm-mesh screen. The retained *Acartia* were rinsed and preserved in a 1:1:8 mix of formalin, glycerol and culture medium with the number of individuals in each life stage group determined as detailed in 4.2.2.1.

Table 4.2.2: Details of the cell densities for the algal species combinations corresponding to the twelve diets assessed for the maintenance of *Acartia* populations required to achieve a 500 µgC L^{-1}. *C- Cryptomonas, I – Isochrysis, N – Nitzschia* and *T – Tetraselmis*

Diet	*Cryptomonas* (cells mL^{-1})	*Isochrysis* (cells mL^{-1})	*Nitzschia* (cells mL^{-1})	*Tetraselmis* (cells mL^{-1})	Total Algal Cell Density (cells mL^{-1})
C	$1.4x10^5$		0	0	$1.4x10^5$
I	0	$2.0x10^5$	0	0	$2.0x10^5$
N	0	0	$1.5x10^5$	0	$1.5x10^5$
T	0	0	0	$5.7x10^4$	$5.7x10^4$
C + I	$6.9x10^4$	$7.4x10^4$	0	0	$1.5x10^5$
C + N	$6.9x10^4$	0	$7.4x10^4$	0	$9.8x10^4$
C + T	$6.9x10^4$	0	0	$2.9x10^4$	$1.7x10^5$
I + N	0	$9.9x10^4$	$7.4x10^4$	0	$1.3x10^5$
I + T	0	$9.9x10^4$	0	$2.9x10^4$	$1.0x10^5$
N + T	0	0	$7.4x10^4$	$2.9x10^4$	$1.4x10^5$
C+I+T	$4.6 x10^4$	$6.6 x10^4$	0	$1.9x10^4$	$1.3x10^5$
C+I+N+T	$3.5x10^4$	$5.0x10^4$	$3.7x10^4$	$1.4x10^4$	$1.4x10^5$

Figure 4.2.1: Photograph of the *Acartia* culture system comprising 500 mL culture unit and 10 L reservoir. Each culture unit is supplied with media from an aerated 10 L header tank, the contents of which were gravity fed through a 2 L hr^{-1} dripper into the culture vessel at a rate of 300 mL hr^{-1}. Excess culture media passed through a 63 µm-mesh screened outflow into a receptacle.

Statistical analysis

All environmental parameters and *Acartia* population data were subjected to Shapiro-Wilk's test for normality and Bartlett's test for homogeneity of variance. Treatment effects in normal data exhibiting homogenous variance were determined by analysis of variance (ANOVA) and Scheffe's multiple means comparison tests.

Data unable to be transformed to meet the assumptions of ANOVA were analysed either using Kruskal-Wallis *k*-sample test, or Mann-Whitney *U*-test where only two treatment levels were analysed. All transformed data were converted back into original units of measurement for presentation in supporting figures and tables.

Culture population density data are reported as the equivalent number of individual *Acartia* L^{-1} to enable comparison between trials conducted in culture units of different volumes.

4.2.3 *Acartia* and golden snapper larvae

The following investigations were designed to assess the effect of small volume aquaria (3.5 L) and larval stocking density on the survival of golden snapper larvae with a view to investigating the growth and survival of golden snapper when fed life stages of *Acartia*. Survival of golden snapper larvae had only been successful in volumes larger than 1 m^3, the greatest success achieved in 40 m^3 volumes (Glenn Schipp, DAC, *pers. comm.*). Larval survival in small volumes would enable replication of treatments within a shorter time frame, and result in more efficient use of spatial, temporal and consumable resources.

Common methods

Detailed below are the techniques and protocols used in the production of golden snapper larvae, the culture and handling of the small-strain tropical rotifer and *Acartia* life stages, and the experimental aquarium systems used in subsequent trials.

Golden snapper production

Larvae of golden snapper were obtained from broodstock held at DAC using techniques similar to those used with barramundi detailed in the previous chapter (Section 3.2.3). Female golden snapper would exhibit serial spawning over four successive nights. The golden snapper larvae were hatched in 1m^3 larval rearing tanks supplied with 1 µm filtered, ultra-violet sterilised seawater at 35 ‰.

Acartia culture

Acartia were maintained in an aerated 150 L conical tank in a light *Tetraselmis* culture (~10^4 cells mL^{-1}) at 30 ‰. The tank was fitted with a 150 µm mesh screen at 30 cm above the base of the cone. Nauplii were harvested daily by turning off the aeration and allowing the culture to settle. Nauplii were then concentrated at

the base of the cone using a torch. *Acartia* copepodids were too large to pass through the screen and were excluded from the harvest. After ten minutes the lower valve in the conical tank was opened to allow the nauplii contained in the 8 L of culture media below the screen to be collected in a submerged 60 µm mesh hand net.

The collected nauplii were subsequently rinsed gently with 1 µm filtered, ultraviolet-treated seawater at 30 ‰. The appropriate volume of concentrated, rinsed *Acartia* nauplii was transferred to each of the aquaria using a 100 mL beaker to achieve a density of 5 nauplii mL^{-1}.

The culture system was housed indoors in the larval rearing area of the DAC hatchery. Photoperiod was artificially extended to 13 L:11D using florescent lights which maintained light levels between 450 and 550 lux (8.8 and 10.7 µmol s^{-1} m^{-2}).

Water quality assessment

Temperature (°C), salinity (‰), pH, dissolved oxygen (DO, mg L^{-1}), nitrite (mg NO L^{-1}), and ammonia (mg NH_3N L^{-1}) were monitored daily using equipment described in the Common Methods of Section 3.2. Water exchanges were conducted when nitrite or ammonia were detected at levels approaching 0.10 mg NH_3N L^{-1} using the appropriate HACH colourimetric tests.

Aquarium setup

Fifteen 4.25 L cylindrical plastic aquaria were spray painted black on the outside to simulate conditions in the $1m^3$ black polycarbonate larval rearing tanks. Each aquarium was filled with 3.5 L of ultra-violet 1 µm filtered seawater at 30 ‰ and *Isochrysis* at $2.5x10^4$ cells mL^{-1}. The aquaria were in turn were placed in a 150 L Reln® tank water bath as depicted in Figure 4.2.2 to minimise diel temperature fluctuations.

Each aquarium was fitted with a 25 mm diameter PVC centre pipe covered with 150 µm mesh screen to prevent golden snapper larvae from entering the centre-pipe and being removed from the aquaria by the siphon (Figure 4.2.3). An air stone was placed inside the centre pipe to provide gentle aeration without disturbing the golden snapper larvae.

Water exchanges were conducted by simultaneously siphoning culture water from within the centre screen while replenishing the same volume of new water via a gravity-fed dripper system. Supported above the Reln® tank water bath was a series of reservoirs fitted with a regulated siphon that fed directly into each aquarium.

Figure 4.2.2: Larval rearing system comprising eighteen 3.5 L aquaria fitted with centre pipe and gravity feed water exchange, supplementary lighting and aeration. Four 25 L buckets served as exchange seawater reservoirs.

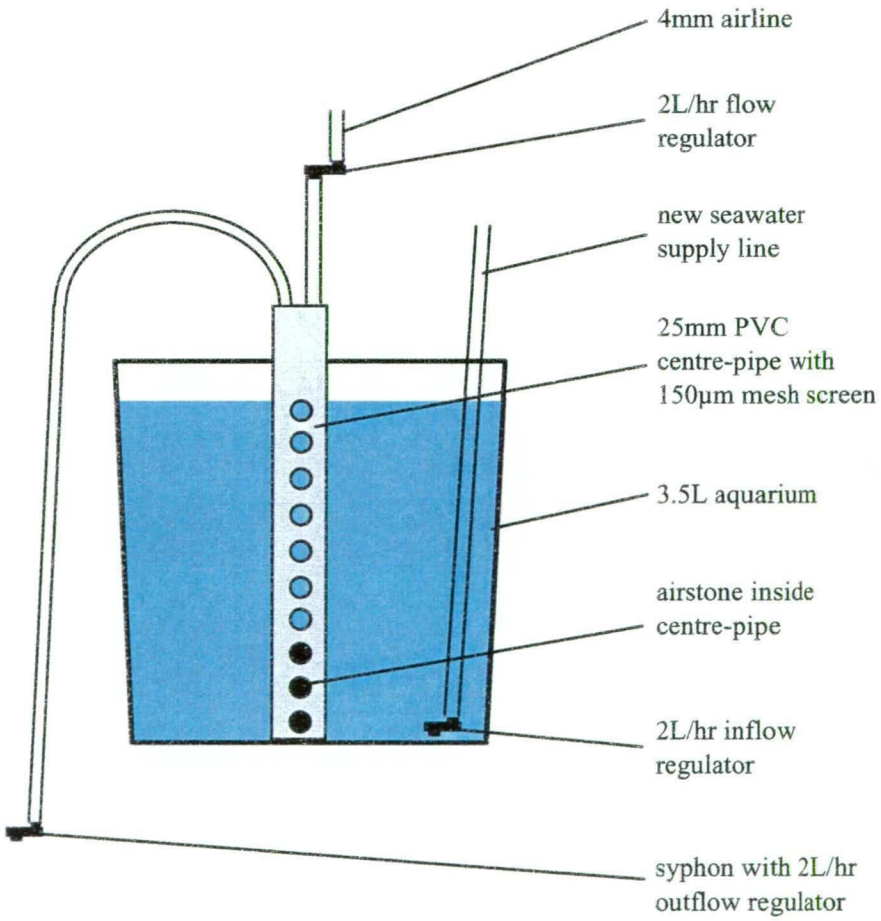


Figure 4.2.3: Diagram of an individual 3.5 L aquarium with centre-pipe. A centre-pipe facilitating water exchange and provision of aeration

Survival of golden snapper larvae in the first trial conducted using the system was extremely poor at 0% (Appendix C8). Aquarium design was a possible confounding factor and an alternative design was included in which the centre pipe was removed, and replaced with a regulated air-supply line and 44 µm mesh screened outlet at the water surface which maintained the 3.5 L volume (Figure 4.2.4).

New water was introduced to the test aquaria via the same gravity feed system as the original aquaria, with excess water exiting the system via an outlet covered in a 44 µm mesh screen located at the 3.5 L water level.

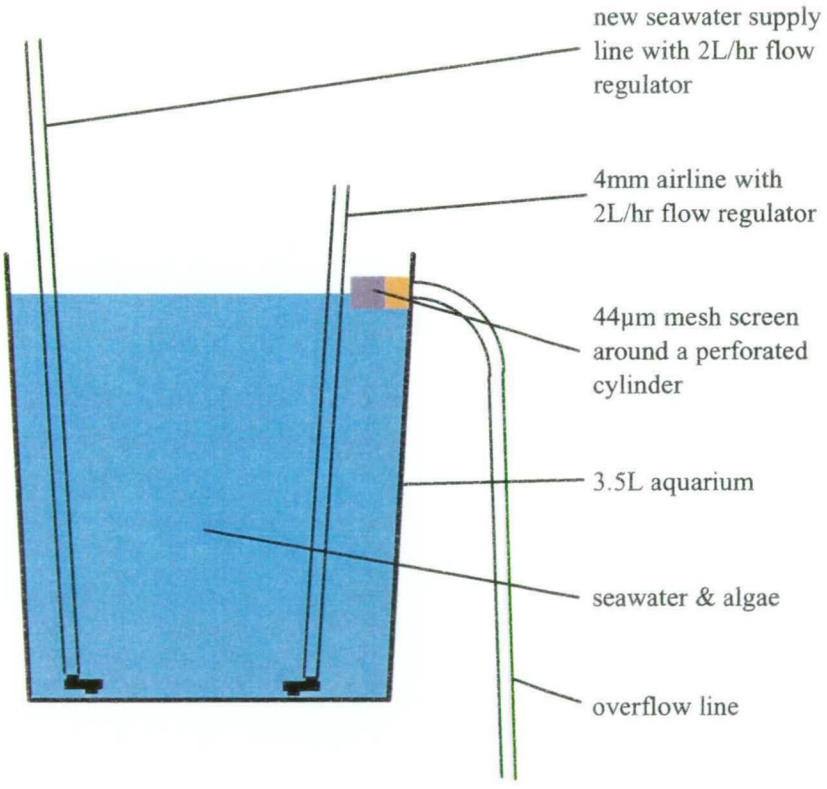


Figure 4.2.4: Alternative aquarium design. The centre-pipe of the original 3.5 L aquarium has been removed and replaced with a regulated air supply line and a screened outlet.

4.2.3.1 Survival of golden snapper larvae in small volume aquaria as influenced by larval stocking density

Survival of golden snapper larvae was reportedly low in small volumes less than one litre (Glenn Schipp, DAC, *pers. comm.*). The need for replication of treatments in the assessment of larval performance favours the use of small volume experimental systems to facilitate the most efficient use of available resources investigating larval performance when fed copepod life stages as opposed to rotifers and *Artemia*.

The following trial was designed to assess the influence of larval survival in 3.5 L aquaria as affected by larval stocking density. A second component was incorporated in the trial to assess the effect of aquarium design on larval performance. The alternative aquarium was designed to minimise stress levels experienced by larvae during water exchange and monitoring procedures.

Fifteen aquaria with centre-pipes and three aquaria of alternative design lacking centre-pipes were set up as described. All aquaria were filled with 3.5 L of 1 µm filtered, ultra-violet treated seawater at 30 ‰ and *Isochrysis* at 2.5×10^4 cells mL^{-1}.

Each aquarium was inoculated with golden snapper larvae early in the morning of day 2 post-hatch under low light conditions prior to the completion of yolk-sac absorption and the commencement of exogenous feeding. All larvae were transferred using a 50 mL beaker to minimise handling stress.

Three replicate aquaria with centre pipes were inoculated at each of the five densities: 5, 10, 20, 35 or 50 larvae L^{-1}. The three aquaria of alternative design were all inoculated at 20 larvae L^{-1}.

The trial was inoculated with golden snapper larvae hatching from eggs released on the third night of serial spawning in an attempt to maximise chances of larval survival. Larvae developing from eggs released on the second and third nights of the spawning cycle tend to be healthier (Lim et al., 1985a).

Recently harvested *Acartia* nauplii were presented at a density of 3 nauplii mL^{-1}.

Golden snapper larvae were visible in the water column the day following inoculation (3 dph), and live food densities adjusted to 5 nauplii mL^{-1}. The second day after stocking, few 4 dph larvae were visible. All aquaria were drained through a 44 µm mesh screen. The numbers of living and dead larvae were counted for each aquarium, and any live larvae were measured and larval gut contents assessed.

4.3 Results

4.3.1 Life cycle and demographics

4.3.1.1 Life cycle

Twelve distinct stages were identified in the life cycle of *Acartia*. *Acartia* are broadcast spawners and the resultant spherical eggs, approximately 90 µm in diameter, are negatively buoyant. The eggs of *Acartia* appear to be sticky, rendering them difficult to work with.

The first naupliar stages of *Acartia* are initially oval in shape with a flattening of the anterior and posterior margins. Progressive development through the remaining five stages corresponds to elongation of the nauplius body producing a more angular-ovate shape as indicated by the increase in length to width ratio (Figure 4.3.1 and Table 4.3.1). The naupliar body of *Acartia* is ovate, not dorso-ventrally flattened, and is generally unpigmented except for the 'nauplius eye' - a red pigment spot located anteriorly on the dorsal surface. In the later naupliar stages, the rudimentary antennules resemble "horns" protruding anteriorly from the rostral region.

Metamorphosis from the final stage nauplius NVI to first stage copepodid CI results in a copepodid with an elongated prosome comprising three quarters of the total body length. The anterior region of the cephalosome is angular in appearance. Elongation of the copepod continues post-metamorphosis through the following six copepodid stages corresponding to distinct increments in total length (Figure 4.3.2 and Table 4.3.2).

Sexual dimorphism is evident at copepodid stages CIV and CV under high magnification using a compound microscope. However reliable, rapid differentiation of gender using high power (6.4x) on a dissecting microscope is generally not possible until copepodid stage CVI unless the operator has considerable prior experience with the copepod.

Adult (CVI) male and female *Acartia* differ in total body length, antennule morphology, fifth periopod morphology and urosomal segmentation (Figure 4.3.3). Adult female *Acartia* are 14% longer than adult males, and females possess a urosome comprising four somites compared with five in males.

The female urosome is short and stout, approximately one-quarter the length of the female cephalothorax. The male urosome is approximately one-third the length of the cephalosome. The obviously larger genital pore of the female comprises two fused somites. The right antennule of male *Acartia* is geniculate, with both antennules marginally more stout than the non-geniculate female antennules. The fifth pair of periopods of the mature male are also obviously specialised resembling an asymmetrical claw relating to their role during copulation. The male fifth

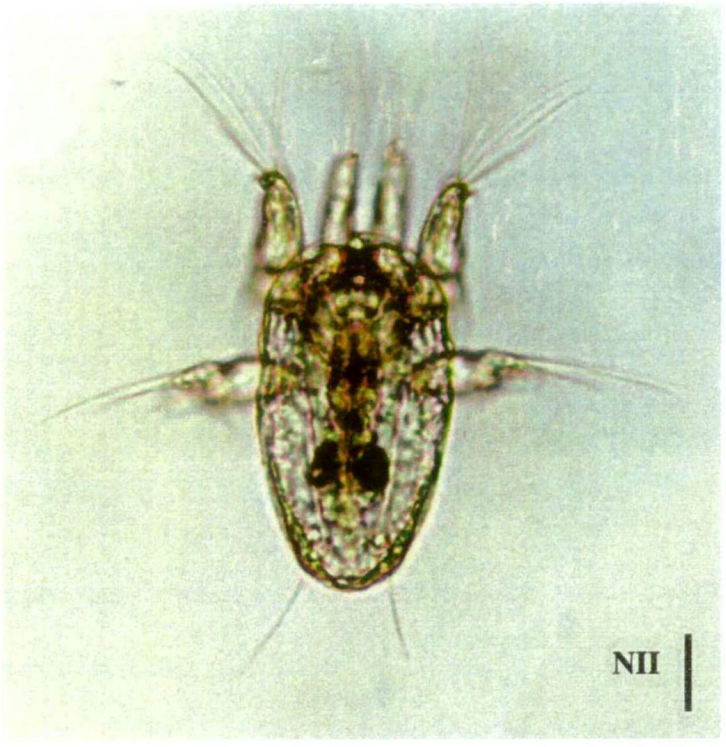
periopod pair is markedly different from the symmetrical and simple fifth periopod of the adult female.

Table 4.3.1: Dimensions (mean ± standard error) of the six naupliar stages of *Acartia*. L - nauplius length measured at the longest point from the posterior to anterior, and W - width measured at the widest point. L:W is the ratio of length to width. n indicates sample size.

Life Stage	L ±SE (µm)	W ±SE (µm)	n	L:W
N I	107 ±1	63 ±1	30	1.7
N II	134 ±1	70 ±1	30	1.9
N III	152 ±2	79 ±2	25	1.9
N IV	174 ±1	87 ±1	30	2.0
N V	201 ±2	111 ±4	25	1.8
N VI	248 ±4	118 ±3	30	2.1

Table 4.3.2: Dimensions (mean ± standard error) of the six copepodid stages of *Acartia*. L - total body length measured from the rostrum to the caudal furca. W - cephalosome width measured at the widest point. L:W is the ratio between total length and width. n indicates sample size.

Life Stage	L ±SE (µm)	W ±SE (µm)	n	L:W
C I	392 ± 8	174 ±4	14	2.3
C II	446 ± 5	195 ±2	25	2.3
C III	542 ± 6	221 ±3	19	2.5
C IV	611 ±22	247 ±6	5	2.5
C V m	675 ±12	265 ±3	16	2.5
C V f	683 ± 8	263 ±5	19	2.6
C VI Male	777 ± 4	288 ±3	28	2.7
C VI Female	889 ± 6	333 ±3	26	2.7



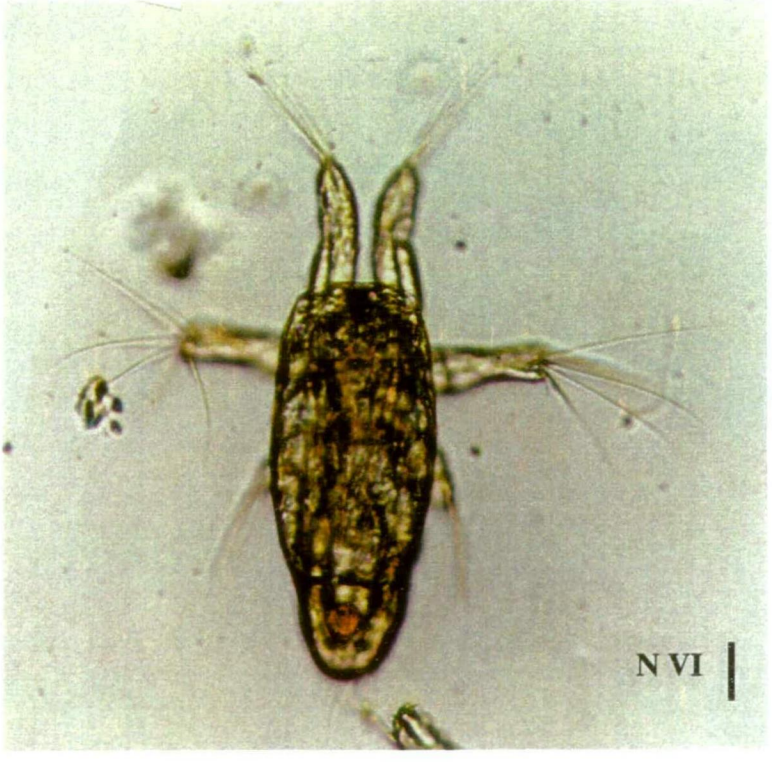


Figure 4.3.1: Photomicrographs of two representative nauplius stages (NII and NVI) of the Northern Territory isolate of *Acartia*. Scale bars are 50 µm.

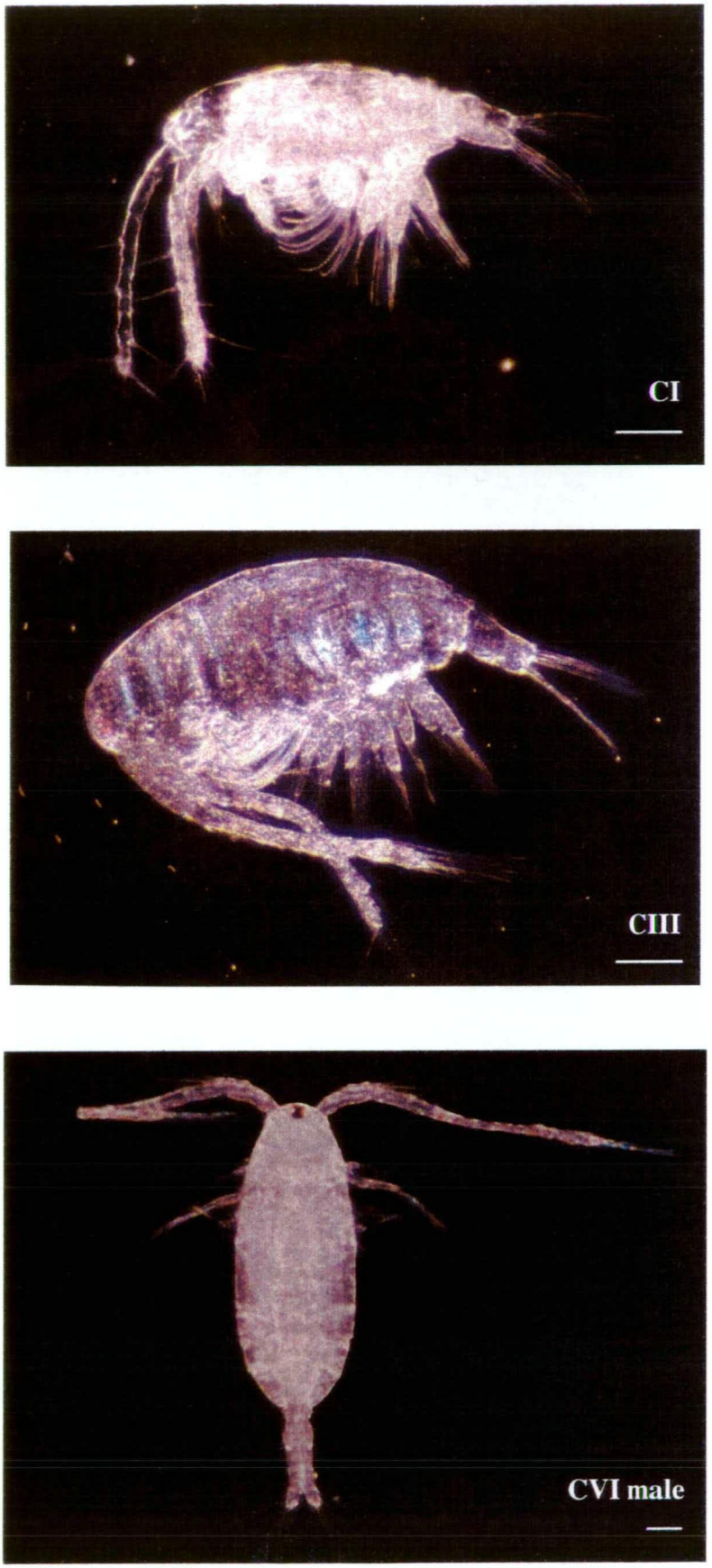
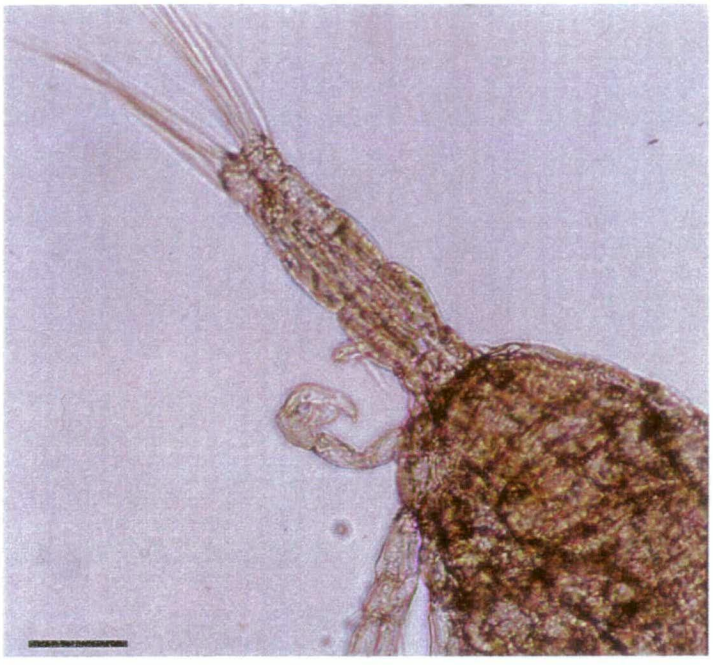
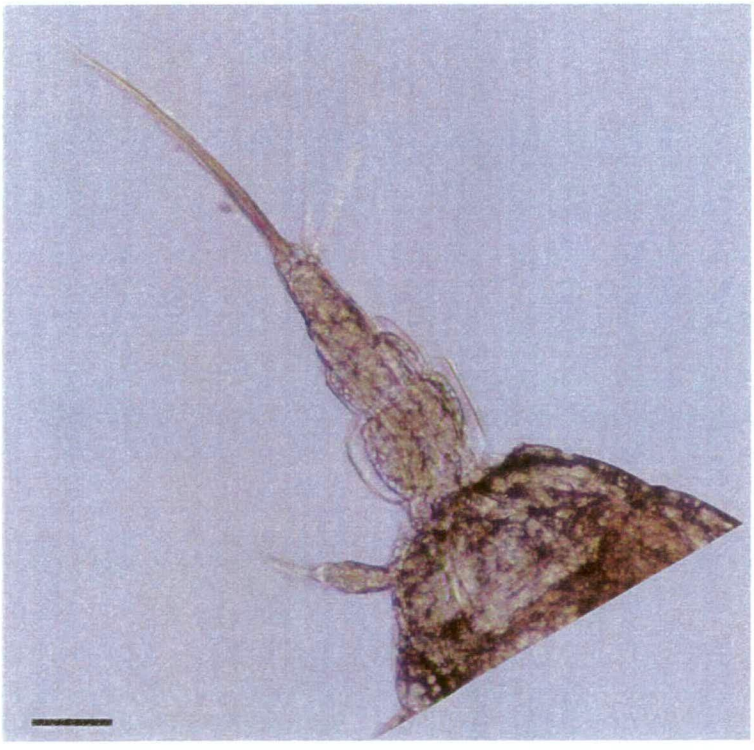


Figure 4.3.2: Photomicrographs of representative copepodid stages of the Northern Territory *Acartia*. Scale bars are 100 µm.



(a)



(b)

Figure 4.3.3: Photomicrographs illustrating sexual dimorphism between the urosome and fifth peripod of mature (a) male and (b) female copepodid stage CVI of *Acartia*. Scale bars are 100 µm.

4.3.1.2 Demographics and productivity

Acartia cultures exhibited a mean generation time in the order of eight days when maintained at 29.4 °C and 35.4 ‰ and fed a diet of *Rhodomonas* and *Tetraselmis*.

A peak in population density of 210 *Acartia* L^{-1} was recorded on day four of culture followed by a decline reaching a low of 56 individual L^{-1} before completion of the culture medium exchange. A further decline to 5 individuals L^{-1} on day 14 was observed which increased to 76 *Acartia* L^{-1} by day 16 of the trial. The increase in contaminant rotifer numbers above 11.5 L^{-1} was coincident with the decline in *Acartia* numbers (Table 4.3.3).

Table 4.3.3: *Acartia* and rotifer densities (individuals L^{-1}) recorded for cultures fed *Rhodomonas* and *Tetraselmis* for a period of 16 days and exposed to an average temperature of 29.4 °C and salinity of 35 ‰.

Day	*Acartia* L^{-1} (mean ±SE)	rotifers L^{-1} (mean ±SE)	Day	*Acartia* L^{-1} (mean ±SE)	rotifers L^{-1} (mean ±SE)
1	39.5 ±6.95	0.0 ±0.00	10	22.0 ±5.60	33.0 ±9.11
2	85.0 ±9.26	1.5 ±0.50	11	10.5 ±3.59	82.0 ±27.5
3	129 ±25.3	1.0 ±0.58	12	6.5 ±2.36	77.5 ±45.6
4	160 ±18.7	9.5 ±1.26	13	15.0 ±4.20	151 ±34.3
6	128 ±24.4	11.5 ±2.87	14	4.5 ±2.22	328 ±71.6
7	44.0 ±18.0	79.0 ±12.5	15	6.0 ±1.83	728 ±220
8	55.5 ±19.9	106.5 ±55.4	16	76.0 ±22.1	14700 ±4630

Population composition data obtained daily proved extremely variable over the sixteen days of culture. The total numbers of *Acartia* obtained in the 500 mL samples were small and not all life stages were present in all samples. To obtain some insight into the population composition on a daily basis individual stages were grouped into the categories: eggs, early nauplii (NI-NIII), late nauplii (NIV-NVI), immature copepodids (CI-CV) and adults (CVI males and females). No clear relationship between the abundance of each demographic stage could be seen between the five groups in terms of their respective abundance over time, the most numerous categories across all samples being the early nauplii which were most numerous on day three (Figure 4.3.4). The numeric abundance of early nauplii greatly exceeded the number of eggs sampled the preceding day. Similarly, the abundance of subsequent demographic groups was observed to decline. The loss of samples collected on days five and nine of the culture sequence may have masked any transition of abundance between late nauplii and early copepodid stages.

The duration of each life stage is not immediately apparent from the data collected however it would seem that progression through early nauplius stages is in the order of one stage per day. Progression through the late nauplius stages is approximately one stage per two days. Similarly, development through the six copepodid stages is in the order of three days. The marked increase in CVI copepodids on day 16 was most probably an artifact of sampling as the entire 10 L was sampled on the final day with recorded abundances converted to the number of individuals in 500 mL. The observed decline in egg production may be a direct reflection of competition of *Acartia* copepodids with rotifers for resources.

The temperature, salinity, dissolved oxygen, pH and light levels recorded for the replicate *Acartia* cultures were not significantly different over the duration of the sixteen-day trial, with mean levels and ranges presented in Table 4.3.4.

Table 4.3.4: Environmental parameter mean values (mean ± standard deviation) and range recorded for the duration of the sixteen-day trial with *Acartia.*

Parameter	Mean ±SE	Minimum	Maximum
Temperature (°C)	29.4 ±0.65	27.0	30.4
Salinity (‰)	35.4 ±0.74	35.0	37.5
pH	8.1 ±0.13	7.86	8.49
DO (mgO_2L^{-1})	6.3 ±0.67	5.22	10.2
Light (lux)	23 ±2.6	14	29

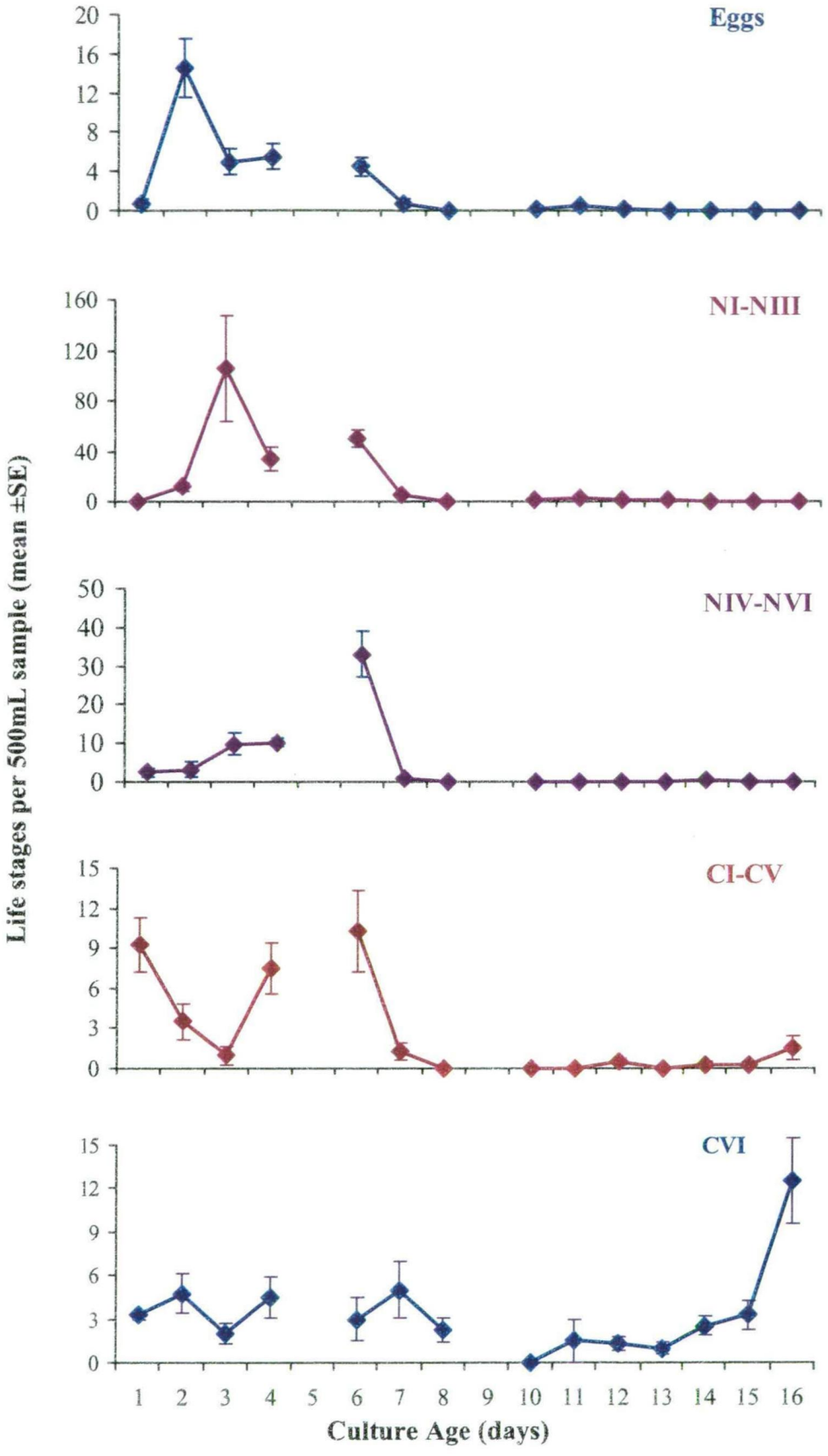


Figure 4.3.4: Mean demographic composition recorded for *Acartia* culture populations maintained on a diet of *Rhodomonas* and *Tetraselmis* for a period of 16 days at 29.5 °C and 35 ‰.

4.3.2 Culture of *Acartia*

4.3.2.1 The effects of light source and culture volume

Moderate light intensity and larger volume cultures promoted growth in *Acartia*. Culture volume, light source and light intensity all exerted a significant influence on the total number of *Acartia* life stages developing in 500 mL culture units over five days ($p<0.05$, Table 4.3.6). The total number of *Acartia* developing under high intensity artificial light conditions did not differ significantly from that developing under both the low and high natural light intensities. All three light conditions produced an increase from an initial stocking density of 120 individuals L^{-1} to the equivalent of 700 *Acartia* L^{-1} in five days. Overall *Acartia* culture populations were more productive when maintained in 500 mL volumes than 150 mL (Table 4.3.5).

Both the number of copepodids and the number of nauplii developing during the five days showed a similar trend. The number of copepodids developing under natural light conditions in 500 mL cultures corresponded to densities of around 555 individuals L^{-1} (Figure 4.3.5a) were significantly different from all other cultures. Copepodids accounted for approximately two thirds of the total *Acartia* population. Nauplii were significantly more numerous in 500 mL cultures exposed to natural light and high intensity artificial light being present at a mean density of 225 L^{-1} (Figure 4.3.5b).

The conditions of high intensity, artificial light yielded a significantly greater number of eggs (480 L^{-1}) than any other combination of light source, intensity or culture volume (Figure 4.3.5c).

All *Acartia* cultures experienced the same temperature or salinity ranges which were not influenced by light source, light intensity or culture volume treatment factors. Temperature ranged from 29.0 °C through to 32.8 °C, with a mean of 30.7 ±0.17 °C. Salinity ranged from 30 ‰ through to 37 ‰ corresponding to a mean of 34.8 ±0.21 ‰. A trend toward increased salinity with increasing light intensity was evident.

Light source and light level treatments produced significantly different light intensities (Table 4.3.6) with light source associated with significant differences ($p<0.05$) in *Acartia* culture pH and dissolved oxygen levels. Cultures maintained under artificial light conditions had associated lower pH and dissolved oxygen levels (Table 4.3.6). No significant environmental effects were attributable to the influence of culture volume.

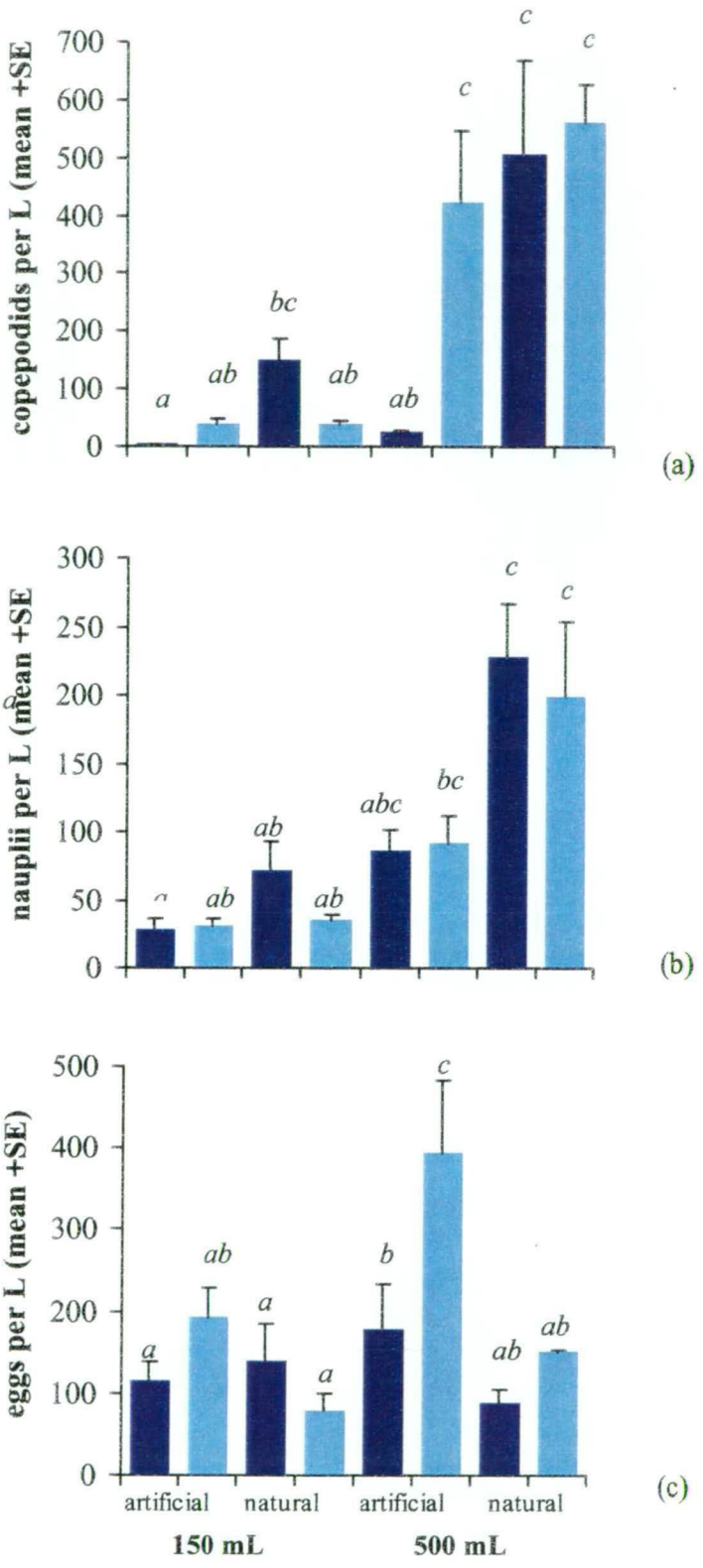


Figure 4.3.5: Number of *Acartia* a) copepodids CI through CVI, b) nauplii NI through NVI, and c) eggs present after 5 days culture under different light source, light intensity (■ low and ■ high) and culture volume conditions. Italicised superscripts indicate a significance differnce at $p<0.05$ as identified using ANOVA and Scheffe's multiple means comparison of square root transformed data.

Table 4.3.5: Total number of *Acartia* reported as individuals L^{-1} developing over five days as influenced by light source, light intensity and culture volume. Scripts indicate a significance difference at $p<0.01$ detected by ANOVA and identified by Scheffe's multiple means comparisons.

Light Source	Light Level	Mean ±SE	Minimum	Maximum	$p<0.01$
150 mL					
Artificial	Low	15 ±8	0	26	*a*
Artificial	High	34 ±9	8	62	*a*
Natural	Low	111 ±85	37	209	*bc*
Natural	High	36 ±20	23	48	*ac*
500 mL					
Artificial	Low	55 ±34	48	61	*a*
Artificial	High	257 ±233	183	330	*b*
Natural	Low	367 ±303	306	428	*b*
Natural	High	380 ±264	373	386	*b*

Table 4.3.6: Mean light intensity (lux) in relation to light source and light level treatments experienced by *Acartia* cultures. Differing treatment means were detected by ANOVA and identified by Scheffe's multiple means comparisons significant at $p<0.0001$.

Light Source	Level	Mean	SE	Minimum	Maximum	$p<0.0001$
Artificial	Low	36	1.7	30	43	*ab*
Artificial	High	496	22.4	400	660	*bc*
Natural	Low	32,810	1,370	24,700	37,500	*c*
Natural	High	62,900	4,350	32,000	79,000	*d*

Table 4.3.7: Relationship between *Acartia* culture pH, dissolved oxygen (DO) and light source/level. Significant differences ($p<0.05$) were detected by ANOVA and identified Scheffe's multiple means comparison test. Different scripts indicates significant differences.

Light	pH (mean ±SE)	DO mgL^{-1} (mean ±SE)	DO % (mean ±SE)	$p<0.05$
Artificial Low	8.0 ±0.06	6.5 ±0.30	85 ±5.6	*a*
Artificial High	8.1 ±0.04	7.7 ±0.22	105 ±2.0	*a*
Natural Low	8.7 ±0.07	10.2 ±0.67	139 ±8.2	*b*
Natural High	8.6 ±0.09	10.8 ±0.90	142 ±10	*b*

4.3.2.2 The effect of stocking density

Inoculation of cultures at an initial density equivalent to 80 to 160 *Acartia* copepodids L^{-1} would appear to achieve most consistent population increases. Initial stocking density was not observed to influence *Acartia* culture productivity significantly in terms of the number of nauplii, copepodids, adults or sex ratio ($p>0.05$). Egg density data did however exhibit a response to initial stocking density (Figure 4.3.6).

Large variations were evident within each treatment after seven days culture within each treatment level (Table 4.3.8). All data exhibited non-normal distribution and heterogeneous variance, and so were analysed using Kruskal-Wallis *k*-sample test.

The average number of eggs recorded from cultures inoculated at 80 *Acartia* L^{-1} and 160 *Acartia* L^{-1} was 165 ±75 eggs L^{-1}, between two and twelve times more than that determined for the other densities investigated.

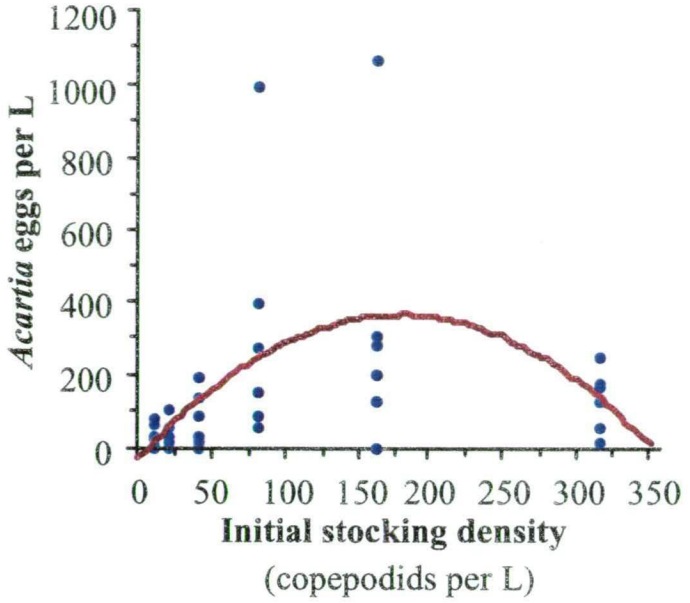
Environmental parameters were consistent across all 36 *Acartia* cultures (Table 4.4.10). The increase in salinity from 35 ‰ to 41 ‰ may be attributable to the evaporation experienced by cultures as a result of the constant temperature units in the laboratory.

Table 4.3.8: Total number of *Acartia* developing over the five days, the corresponding net increase and sex ratio for cultures inoculated at the various densities. (Imm C indicates only immature copepodids were present after five days.)

Density	*Acartia* L^{-1} (mean ±SE)	Net Increase (*Acartia* L^{-1})	Sex Ratio (mean ±SE)
10	50 ±20	40	imm C
20	220 ±80	200	imm C
40	300 ±200	260	2.8 ±0.0
80	1,080 ±470	1,000	2.0 ±2.0
160	1,120 ±640	960	2.6 ±2.3
320	385 ±170	65	2.2 ±2.5

Table 4.3.9: Environmental conditions measured in *Acartia* cultures during the trial assessing the effect of stocking density on population growth.

Parameter	Mean	SE	Max	Max
Temperature (°C)	28.5	0.05	28.0	29.0
Salinity (‰)	41.1	0.15	39.5	43.0
pH	8.74	0.031	8.09	9.01
DO ($mgO_2 L^{-1}$)	28.5	0.28	8.0	16.9
DO (%)	13.0	7.7	102	250



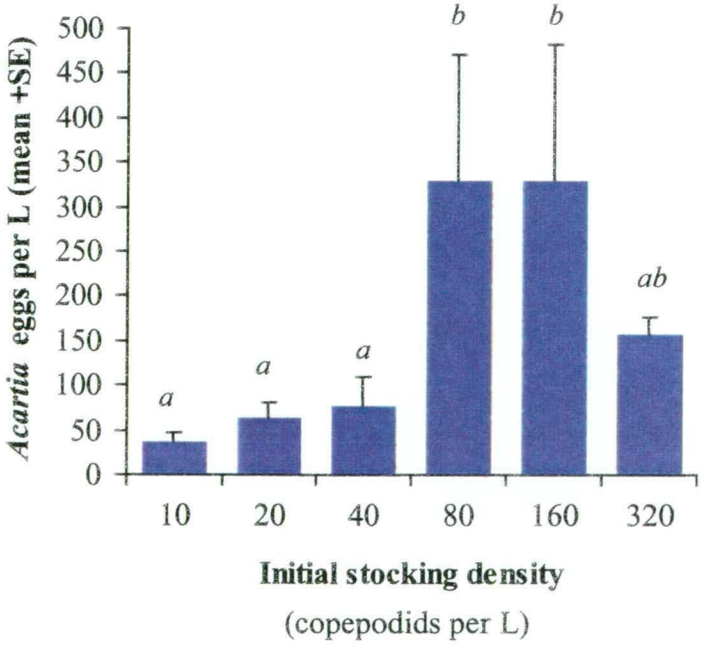


Figure 4.3.6: Effect of stocking density (SD) on the number of eggs produced by *Acartia* cultures maintained on a mixed algal diet for five days. Regression analysis proved significant ($p<0.05$). The equation of the line: *Acartia* eggs = -13.246 + 2.195 SD – 0.006 SD^2; r^2 =0.254. Italicised superscripts indicate significant treatment effects identified by Kruskal-Wallis *k*-sample test in conjunction with Tukey's multiple means comparison.

4.3.2.3 The effects of salinity and temperature

Salinity exerts a strong influence on *Acartia* culture population density masking any temperature effect. The total number of *Acartia* developing from the initial inoculum of 20 copepodids over nine days was significantly influenced by salinity ($p<0.01$). The most dense cultures were those maintained at 32 °C and 35 ‰ yielding population densities equivalent to 860 *Acartia* L^{-1} (Figure 4.3.7). No significant temperature effect on population density was recognised, however the interaction between salinity and temperature proved significant when assessing treatment effects on the numbers of nauplii, copepodids, eggs and total number as well as sex ratio.

The number of eggs recorded after nine days culture was also highest at 35 ‰ and 23 °C with the equivalent of 310 eggs L^{-1} (Figure 4.3.8). The number of *Acartia* nauplii (270 L^{-1}) and copepodids (440 L^{-1}) developing under the various temperature-salinity treatments were also highest at 35 ‰. The greatest number of nauplii equivalent to 470 L^{-1} was recorded at a temperature of 29 °C, with the greatest number of copepodids equivalent to 435 L^{-1} yielded by cultures maintained at 35 °C.

Regression analyses conducted between salinity levels and the number of eggs, nauplii, copepodids and total number of *Acartia* developing in cultures produced parabolic curves with coefficients of determination less than 0.2. The influence of salinity is obvious with the greatest density for each class of *Acartia* occurring at 35 ‰ (Figure 4.3.7). The same analyses conducted using temperature as the independent variable resulted in flatter parabolas with coefficients of determination less than 0.05. The temperature at which each class dominated was related to temperature with 23 °C yielding the greatest number of eggs, 29 °C the most nauplii and 32 °C the greatest density of copepodids (Table 4.3.10). Sex ratios did not differ between treatments, cultures exhibiting a mean ratio of 3.2 ±0.26 females to each male.

Table 4.3.10: Demographic group details (mean ± standard error) for each treatment temperature 23, 26, 29, 32 and 35 °C over the nine day trial.

Treatment	Eggs L^{-1}	Nauplii L^{-1}	Copepodids L^{-1}	Sex Ratio
23 °C	69 ±22	27 ±12	28 ±3.2	3.9 ±0.62
26 °C	57 ±19	71 ±33	38 ±9.3	2.8 ±0.47
29 °C	28 ±6.0	52 ±33.	33 ±14	3.3 ±0.56
32 °C	26 ±9.7	55 ±30	56 ±30	3.0 ±0.41
35 °C	15 ±4.8	22 ±9.6	30 ±13	3.3 ±0.51

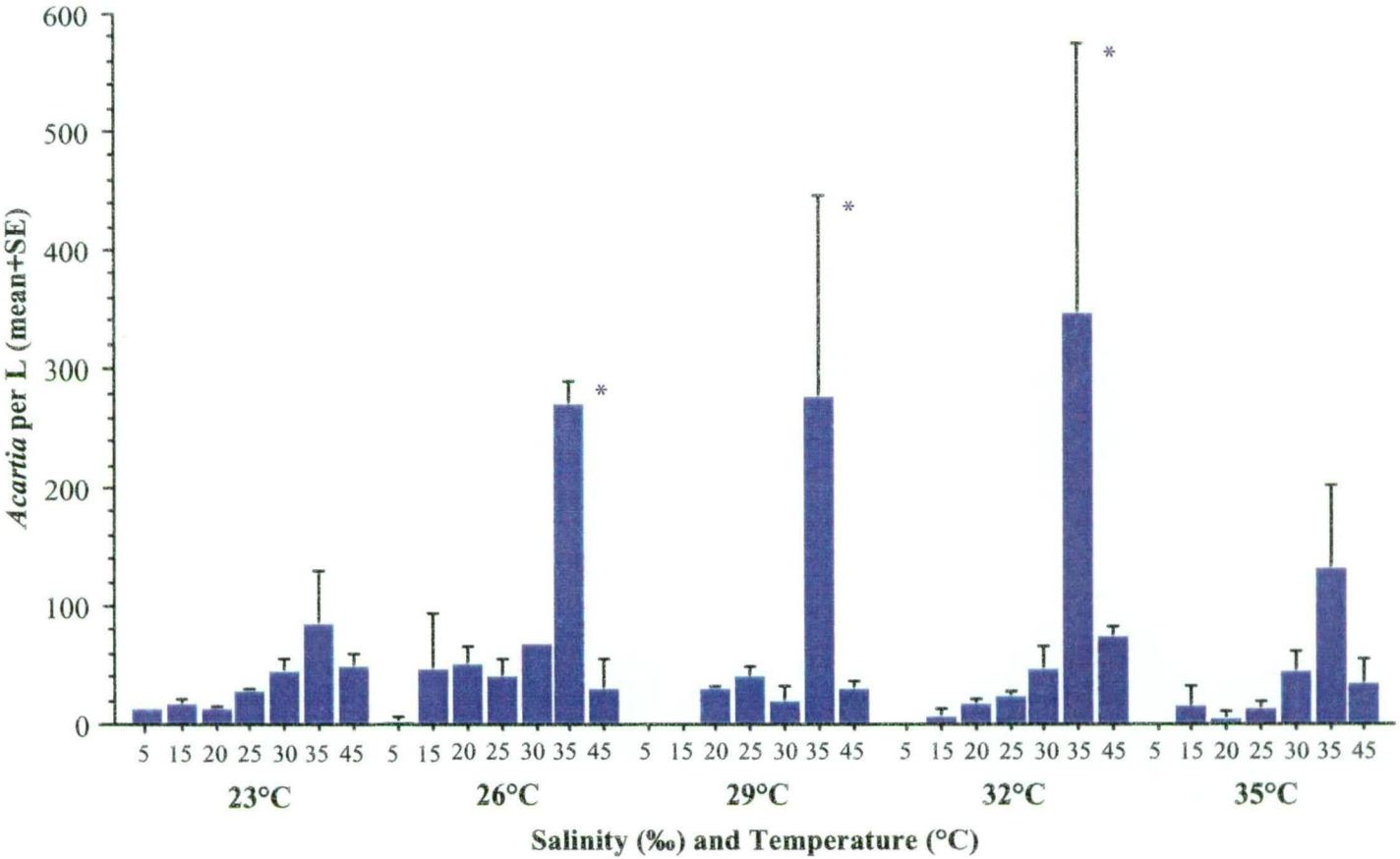


Figure 4.3.7: Influence of temperature and salinity on duplicate 500 mL *Acartia* cultures fed a mixed algal diet as determined by the total number of individuals developing over nine days. Salinity over the range 5 ‰ to 45 ‰ exerted a significant effect, as did the interaction between temperature and salinity. Temperature over the range 23 °C to 35 °C did not significantly affect productivity. * indicates population densities significanlty different from the others as identified by Kruskal-Wallis *k*-sample test ($p<0.01$).

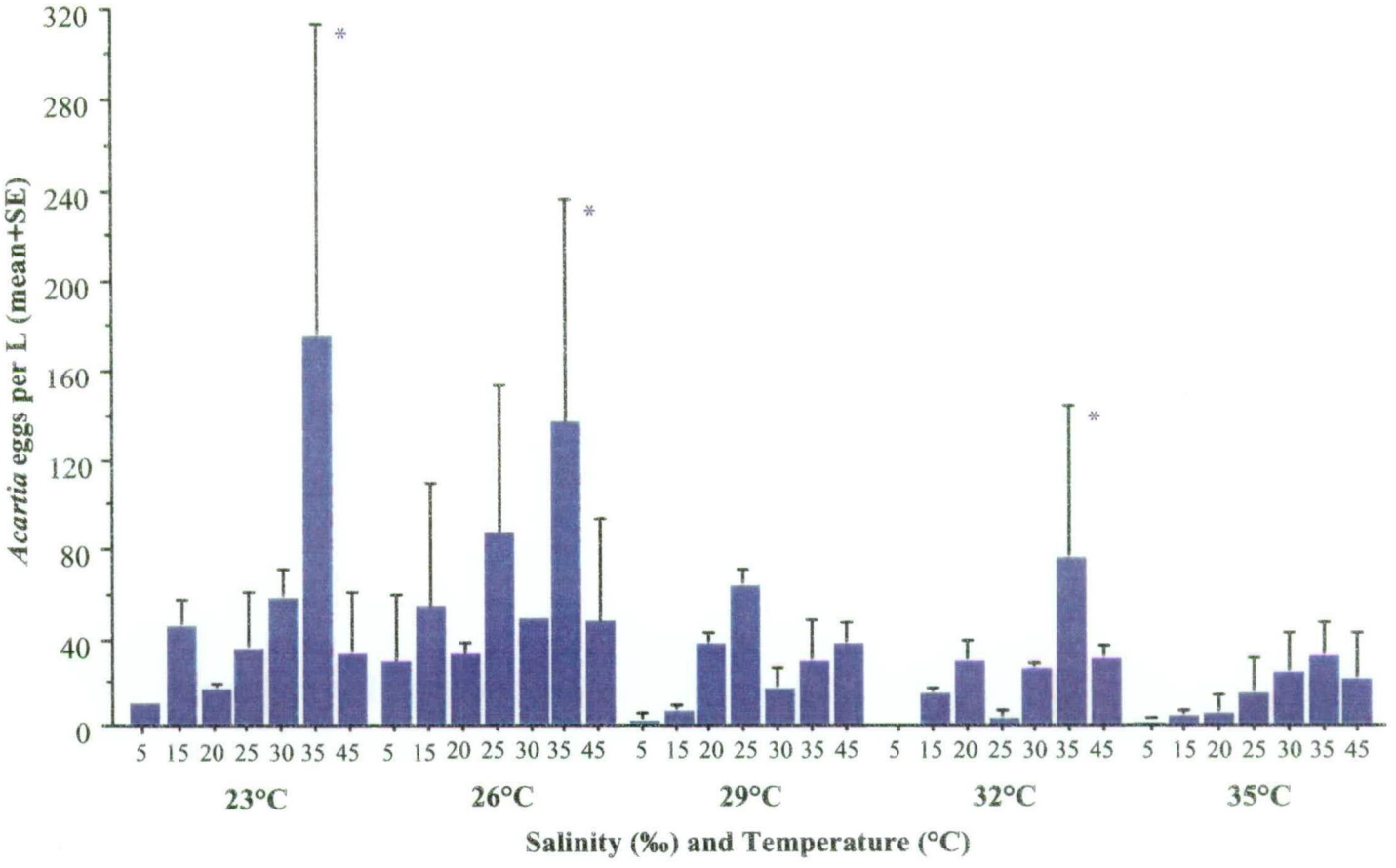


Figure 4.3.8: Influence of temperature and salinity on the number of eggs present in duplicate 500 mL cultures *Acartia* cultures fed a mixed algal diet after nine days. Salinity over the range 5 ‰ to 45 ‰ exerted a significant effect, as did the interaction between temperature and salinity. Temperature over the range 23 °C to 35 °C did not significantly affect productivity. Italicised superscripts indicate significant differences identified by Kruskal-Wallis *k*-sample test ($p<0.01$).

4.3.2.4 The effect of algal species

All diets containing *Isochrysis* generated higher population densities of *Acartia* than the other diets with the exception of the mixed diets comprising three or more algal species. *Acartia* cultures fed *Isochrysis* alone, or in combination with *Nitzschia* or *Tetraselmis*, yielded an average density equivalent to 108 ±20 individuals L^{-1} significantly more than *Acartia* cultures maintained on diets comprising different species or more than three microalgae species. The other diets exhibited a mean culture density of 26 ±5 *Acartia* L^{-1} ($p<0.05$, Figure 4.3.9).

Total numbers of copepodids, nauplii and eggs present after six days exhibited great variation within and between diets. The average density of copepodid stages CI through CVI did not differ significantly ($p>0.05$) between treatments, 13 ±2 copepodids L^{-1} the average with a range from 0 to 36 copepodids L^{-1} recorded. The greatest density of 36 copepodids L^{-1} was recorded from cultures fed *Isochrysis* plus *Tetraselmis*.

The number of nauplii and eggs present after six days were significantly influenced by treatment ($p<0.01$, Figure 4.3.10). Diets containing *Isochrysis* were associated with the higher *Acartia* egg and nauplii densities.

Sex ratio exhibited no significant trend, or significant differences ($p>0.05$), between diets. The mean ratio recorded was 4.0 ±0.5 with a range from 0 to 10.

The temperature during the trial did not differ between treatments exhibiting a mean of 30.9 ±0.05 °C with an associated range from 30.4 °C to 31.4 °C. Similarly, salinity was consistent across all cultures at 35 ‰. Culture media dissolved oxygen (mgO_2 L^{-1}) and pH levels did not differ between treatments with respective means and ranges of 6.6 ±0.09 mgO_2 L^{-1} (5.02 to 9.24 mgO_2 L^{-1}) and 7.9 ±0.02 (7.6 to 8.2).

Review of the diet treatments applied in light of the information obtained from the algal cell density trial documented in Appendix C6 revealed that the actual diets presented to the *Acartia* cultures were in fact approximately less than 1% of the intended algal cell carbon concentration (Table C4.3.11). Diets exhibiting highest population densities were cultures presented diets numerically dominated by *Isochrysis* cells, with the exception of the diet comprising all four species in combination. No relationship was evident between total cell density and *Acartia* population density. Similarly, there was no relationship evident between algal cell carbon concentration (which ranged between 3.9 and 14.7 times the intended levels) and *Acartia* population density or egg production.

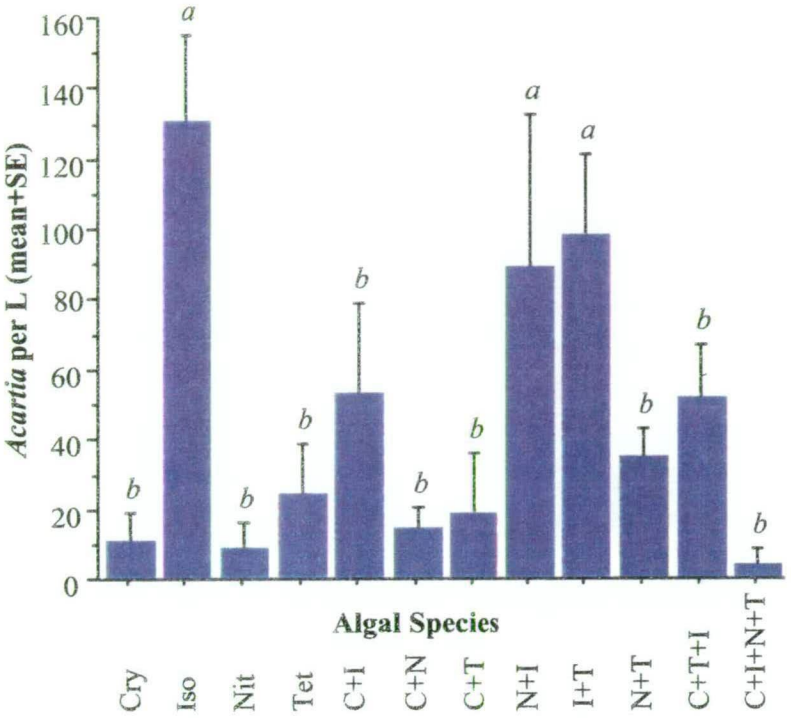
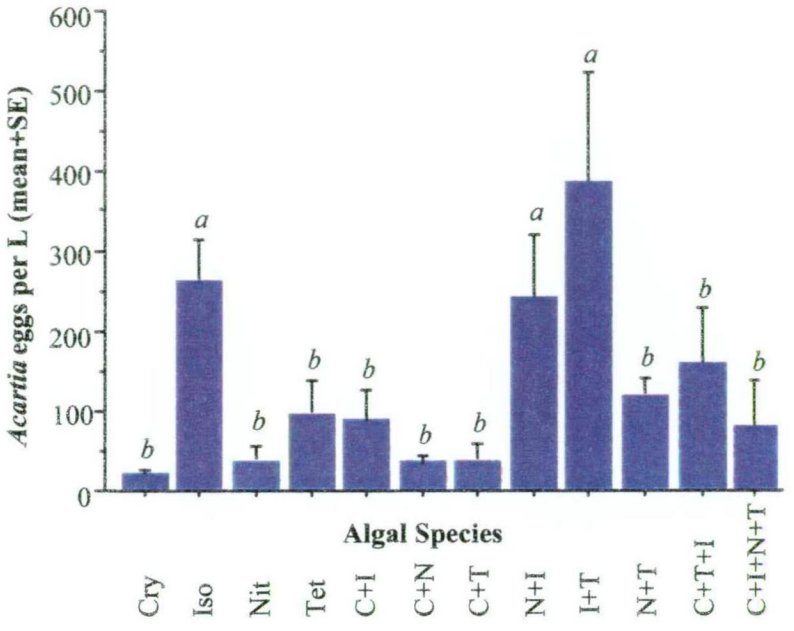
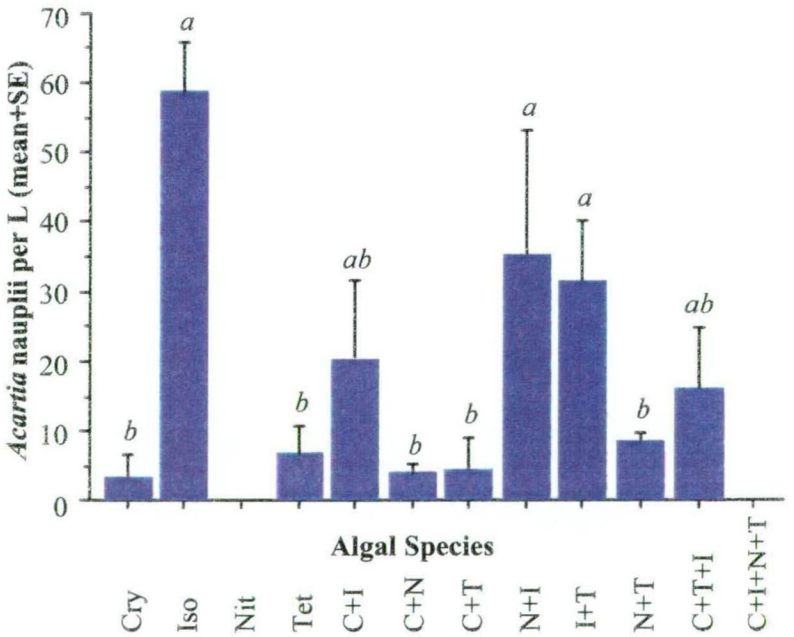


Figure 4.3.9: Influence of diet on the productivity of *Acartia* cultures as indicated by the total number of individuals developing over six days when maintained on various algal diets at 29 °C and 35 ‰. Italicised superscripts indicate significant differences revealed by Kruskal-Wallis *k*-sample analysis and Tukey's multiple means comparison ($p<0.05$).

C – *Cryptomonas*; I – *Isochrysis*; N – *Nitzschia*; T – *Tetraselmis*.



(a)



(b)

Figure 4.3.10: Influence of diet on the productivity of *Acartia* cultures as indicated by the total number of (a) nauplii and (b) eggs present after six days when maintained on various algal diets at 29 °C and 35 ‰. Italicised superscripts indicate significant differences revealed by Kruskal-Wallis *k*-sample analysis and Tukey's multiple means comparison ($p<0.05$).

C – *Cryptomonas*; I – *Isochrysis*; N – *Nitzschia*; T – *Tetraselmis*.

Table 4.3.11: Details of the final combined cell densities, corresponding algal cell carbon concentration and % composition by numerically dominant algae for the algal species combinations corresponding to the twelve diets assessed. C, Cry- *Cryptomonas,* I, Iso – *Isochrysis*, N , Nit– *Nitzschia* and T, Tet – *Tetraselmis*

Diet	Total Algal Cell Density (cells mL^{-1})	Total Algal Cell Carbon Concentration ($\mu gC\ L^{-1}$)	Numerically dominant species
C	$1.4x10^5$	2750	100% Cry
I	$2.0x10^5$	3950	100% Iso
N	$1.5x10^5$	1950	100% Nit
T	$5.7x10^4$	7350	100% Tet
C + I	$1.5x10^5$	2800	52% Iso
C + N	$9.8x10^4$	2300	52% Nit
C + T	$1.7x10^5$	5100	70% Cry
I + N	$1.3x10^5$	2900	57% Iso
I + T	$1.0x10^5$	5700	77% Iso
N + T	$1.4x10^5$	4700	72% Nit
C+I+T	$1.3x10^5$	4650	50% Iso
C+I+N+T	$1.4x10^5$	3950	37% Iso

4.3.3 *Acartia* and golden snapper larvae

Survival of golden snapper was greatest at 6 dph when 2 dph larvae were stocked at starting density of 20 larvae L^{-1} (Figure 4.3.11). Mean survival rates were influenced by aquarium design, with golden snapper stocked into aquaria lacking a centre-pipe (refer to Figure 4.2.4) being 9.7 ±2.46% compared to the 1.5 ±0.86% survival rates exhibited by larvae stocked in aquaria with a centre-pipe (refer to Figure 4.2.3).

All golden snapper larvae retrieved from the aquaria, which measured 3.8 ±0.06 mm in total length, were emaciated with a 'duckbill' appearance to the upper jaw. All the digestive tracts were empty.

The golden snapper retained from the aquaria lacking a centre-pipe exhibited the highest survival rate achieved of 14.3%, that is five times greater than the maximum achieved in any aquaria with a centre-pipe, and they did not exhibit such extreme emaciation, or such a strong a 'duckbilled' appearance despite their digestive tracts also being empty.

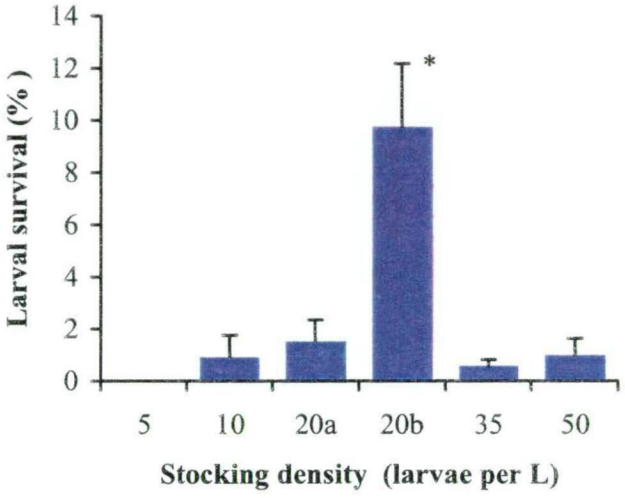


Figure 4.3.11: Larval golden snapper survival as affected by stocking density and aquarium design. At 20 larvae L^{-1} (a) denotes the use of the original aquarium design including a centre-pie, and (b) indicates the use of the alternative aquarium design lacking a centre-pipe. Aquarium design was tested at only one stocking density. * indicates a significantly different mean as identified by ANOVA of arcsine transformed data.

Salinity remained constant during the three days of the trial at 31 ‰ with temperature observed to fluctuate between 30.3 °C and 31.4 °C, ammonia levels increased to 0.035 mg unionised ammonia L^{-1} and nitrite increased to trace levels <0.001 mg nitrite L^{-1}. *Isochrysis* was maintained below 10^4 cells mL^{-1} with feed densities recorded over the range of 0.5 to 4.5 *Acartia* nauplii mL^{-1}.

4.4 Discussion

This study has shown that the Northern Territory species of *Acartia* conforms to the general free-living copepod pattern, progressing through twelve readily distinguishable stages from egg through to ovigerous female over 8 days when maintained at 30 °C, 35 ‰ and fed a microalgal diet comprising *Rhodomonas* and *Tetraselmis*. This calanoid is able to reproduce over a wide range of temperatures, but is restricted in terms of salinity exhibiting a narrow optimum within a few units of 35‰.

From an aquaculture perspective, this copepod meets the criteria for the selection of a larval diet as identified in the general introduction (Section 1.2) presenting life stages of: a) appropriate size, b) occupying the same habitat as the larvae, and c) exhibiting no obvious piscivorous habit.

4.4.1 Life cycle and demographics

This study has shown that the Northern Territory isolate of *Acartia* demonstrated life cycle and demographic characteristics consistent with those reported for Northern Hemisphere and South East Asian species (Greenwood, 1972; Trujillo-Oritz, 1986; Sunyoto et al., 1995; Takahashi & Ohno, 1996; Yoon et al., 1998).

Acartia possesses a life cycle typical of free-living copepods progressing through twelve readily distinguishable stages from egg through to sexual maturity over seven to eight days at 29.4 °C. The recorded lengths for the life stages of *Acartia* exhibit similar trends to those reported for *A. californiensis* (Trujillo-Ortiz, 1986; Figure 4.4.1). *A. californiensis* was cultured at 17 °C compared to 29.5 °C, and as such temperature may be the factor underlying the size disparity between copepodid stages and rate of development (Uye, 1988; Klein Breteler, 1994; Takahashi & Ohno, 1996).

Sexual dimorphism is obvious in *Acartia* from the fourth copepodid stage (CIV) onwards. Females are 13% larger than males, and they exhibit distinctly different antennule morphology; the right antennule of only the adult male is geniculate. Specialisation of the fifth pair of thoracic appendages was observed in both sexes according to published information specific to the genus *Acartia* and generalised reports (Trujillo-Ortiz, 1986; Huys & Boxshall, 1991; Takahashi & Ohno, 1996).

Distinguishing characteristics for each of the twelve *Acartia* stages can be based on length differences in combination with anatomical differences observable under a dissecting microscope (Table 4.4.1). The Darwin *Acartia* exhibits significant potential as a live food as at least four naupliar stages (NI through NIV) exhibit widths of less than 100 µm corresponding to the prey size able to be ingested by marine finfish larvae at the commencement of exogenous feeding (Nellen, 1985; Iglesias et al. 1994; Watanabe & Kiron, 1994) and the greater importance of prey

width than length in determining food particle size suitability for ingestion (Shirota, 1970; Ghan & Sprules, 1993; Fernández-Diaz et al., 1994). The first stage nauplius is also approximately 100 µm in length (mean length recorded as 107 µm, Table 4.3.1), which may in part explain the success experienced in the rearing of lutjanid larvae when presented with *Acartia* nauplii.

From a technical aquaculture perspective it is important that stages be easily identified to assist with assessment of copepod culture health, productivity and harvest.

Numerous researchers have indicated that the eggs dispersed by female *Acartia* are easily handled and separated from the pelagic life stages by siphoning the bottom of culture vessels (for example Støttrup et al., 1986; Sunyoto et al., 1995). My experience and that of other staff at the DAC has proven otherwise; the eggs of the Darwin species of *Acartia* appear to be sticky and adhere to detritus thus rendering their isolation by siphoning near impossible. Difficulty in separating dispersed eggs from detritus and contaminant rotifers in the culture was also reported by Schipp et al. (1999) during the development of their *Acartia* culture system.

The difficulties experienced in isolating stages of *Acartia* and the apparent intolerance of the copepodids to pipetting (Appendix C; C2) precluded the use of the techniques used with *Tisbe*. The creation of artificial cohorts was not possible, resulting in the decision to track the abundance of stages in a mass culture to gain some information on mean generation time and stage durations.

The occurrence of cohorts has been observed in intact culture populations (Ohno & Okamura, 1988) and in wild populations of *Acartia clausi* (Uye, 1982). Ohno et al. (1990) indicated that, as a result of *Acartia* species forming cohorts at relatively short cycles as the common mode of population development, analysis of the population dynamics by cohorts was warranted. This conflicts with the opinion of numerous authors who indicate that cohort analysis is unsuited to the determination of demographic details for continuously reproducing populations with short generation times (for example Kimmerer & McKinnon, 1987; Vijverberg 1989; Saiz et al., 1997) as a result of difficulties in accurately distinguishing between cohorts.

The daily monitoring of *Acartia* indicated a mean generation time (T) in the vicinity of seven to eight days when cultures were maintained at 35 ‰ and 29°C and fed a mixed diet of *Rhodomonas* and *Isochrysis*. This generation time falls within the reported range for tropical *Acartia* (5 to 7 days, Singhagraiwan et al., 1994; 10 days, Sunyoto et al., 1995). Concurrent work with *Acartia* at the DAC based on my preliminary findings indicated a mean generation time within the range of 5 to 7 days depending on culture conditions (Schipp, Bosmans & Marshall, 1999). The shorter time reported by Schipp et al. (1999) is likely to be

due to the absence of rotifers from DAC cultures and subsequent lack of competition for resources, combined with improved water quality as corollary of low rotifer densities. The *Acartia* culture from which the inoculum individuals were obtained for the life cycle investigation exhibited a cyclic fluctuation in total numbers with a frequency of approximately six days.

The low densities observed for the 10 L *Acartia* cultures meant that it was not possible to obtain sufficient numbers in each daily sample to facilitate a comprehensive cohort analysis. The extended mean generation time observed may be due to the confounding influence of rotifers in all the 10 L cultures and their subsequent competition for algae and crowding effects.

The daily *Acartia* population harvest of 500 mL equates to 5% of the total culture, which according to Ohno et al. (1990), should be easily sustained. Ohno et al. (1990) completed an investigation into the level of harvesting tolerated by a self-perpetuating culture of *A. tsuensis*. When chlorophyll levels corresponding to cell densities of $4\text{-}5x10^5$ cells mL^{-1} were maintained, *A. tsuensis* could tolerate up to a 30% daily harvest of the total population. However it should be noted that the presence of rotifers in the 10 L cultures might well have suppressed algal levels sufficiently to adversely affect the ability of the *Acartia* population to sustain the regular low level harvest.

Ohno et al. (1990) further suggested that the ability of *Acartia tsuensis* to resist a declining population under high rates of exploitation could to be related to some or all of the following mechanisms: a) decrease of mortality, b) decline in predation on nauplii by copepodids, c) accelerated recruitment to spawning age owing to accelerated growth, and d) increase in fecundity independent of adult female body size. Observations of *Acartia* culture populations would support their hypothesis (*pers. obs.*).

Sunyoto et al. (1995) reared *Acartia plumosa* at 26-29 °C and 34 ‰ on a diet of *Tetraselmis* at $2\text{-}4x10^7$ cells mL^{-1} reporting a *T* of 10 days, with Ohno & Okamura (1988) working with *A. tsuensis* reporting a *T* of approximately 12 days when cultured at 22-27 °C and 30 ‰. The extended *T* may in part be due to the lower temperatures at which the species were cultured (Uye, 1988; Klein Breteler, 1994; Takahashi & Ohno, 1996), but also may be attributable to the lesser quality single species diets offered (Støttrup et al., 1986; Berggreen et al., 1988; Jónasdóttir, 1994).

The difficulty of isolating *Acartia* eggs from mixed populations had implications for other trials. The apparent ease of isolating inocula of known age and origin afforded by working with a broadcast spawning copepod was not realised with the Darwin *Acartia* species. Isolation of eggs into volumes ranging from 1 mL tissue culture wells, 10 mL to 50 mL volumes in the 70 mL individual culture units used

with *Tisbe*, to 250 mL volumes all failed to support naupliar development beyond one moult, that is if the eggs hatched at all. Similarly, early nauplii individually transferred to the same volume range of individual cultures failed to develop through to copepodids. Copepodids ranging from CI through to CVI failed to survive for extended periods in individual cultures. No egg production was observed from individual *Acartia*. All the individual cultures were supplied with *Isochrysis* and *Tetraselmis* and maintained at 35 ‰ and between 27 and 30 °C under light conditions which subsequently supported populations maintained in 500 mL mass cultures. My assumption is that the handling techniques I used were responsible for the hiatus observed in *Acartia* development in individual cultures.

Table 4.4.1: Features useful in differentiating between life stages of *Acartia*. The length values reported are indicative of relative size differences corresponding to the distance from the rostrum to feruncular setae with descriptions modified from Trujillo-Oritz (1986).

Life Stage	Length (µm)	Identifying Features:
N I	107	▪ nauplius anterior margin blunt ▪ nauplius body approximately the same length as appendages ▪ two short caudal setae visible
N II	134	▪ nauplius body elongates ▪ caudal setae twice the length of previous stage
N III	152	▪ nauplius body elongates further ▪ each caudal seta flanked by shorter spine
N IV	174	▪ appendages one half of nauplius body length
N V	201	▪ appendages one third of nauplius body length
N VI	248	▪ appendages two fifths of the nauplius body length ▪ buds of the first two pairs of swimming legs visible on ventral surface
C I	392	▪ resembles adult, but with only two pairs of swimming legs (periopods) ▪ urosome comprises 1 somite
C II	446	▪ third pair of swimming legs present ▪ urosome somite appears to 'bud'
C III	542	▪ fourth pair of periopods present ▪ second somite develops from 'bud'
C IV	611	▪ fifth pair of periopods appear in rudimentary form ▪ sexual dimorphism evident in urosome ▪ male – three discreet somites visible in urosome ▪ female – two larger somites visible in urosome
C V m	675	▪ sexual dimorphism evident ▪ asymmetrical development of 5th periopod ▪ urosome comprises four somites
C V f	683	▪ symmetrical development of 5th periopod ▪ urosome comprises three somites
C VI M	777	▪ fifth pair of periopods fully developed, segments of both periopods are stout and together form a clasping claw (Figure 4.3.1a) ▪ five somites in urosome ▪ right antennule specialised with geniculation
C VI F	889	▪ fifth pair of periopods fully developed, segments of both are more symmetrical, slender and do not form a clasping claw (Figure 4.3.1b) ▪ four somites in urosome ▪ antennules symmetrical

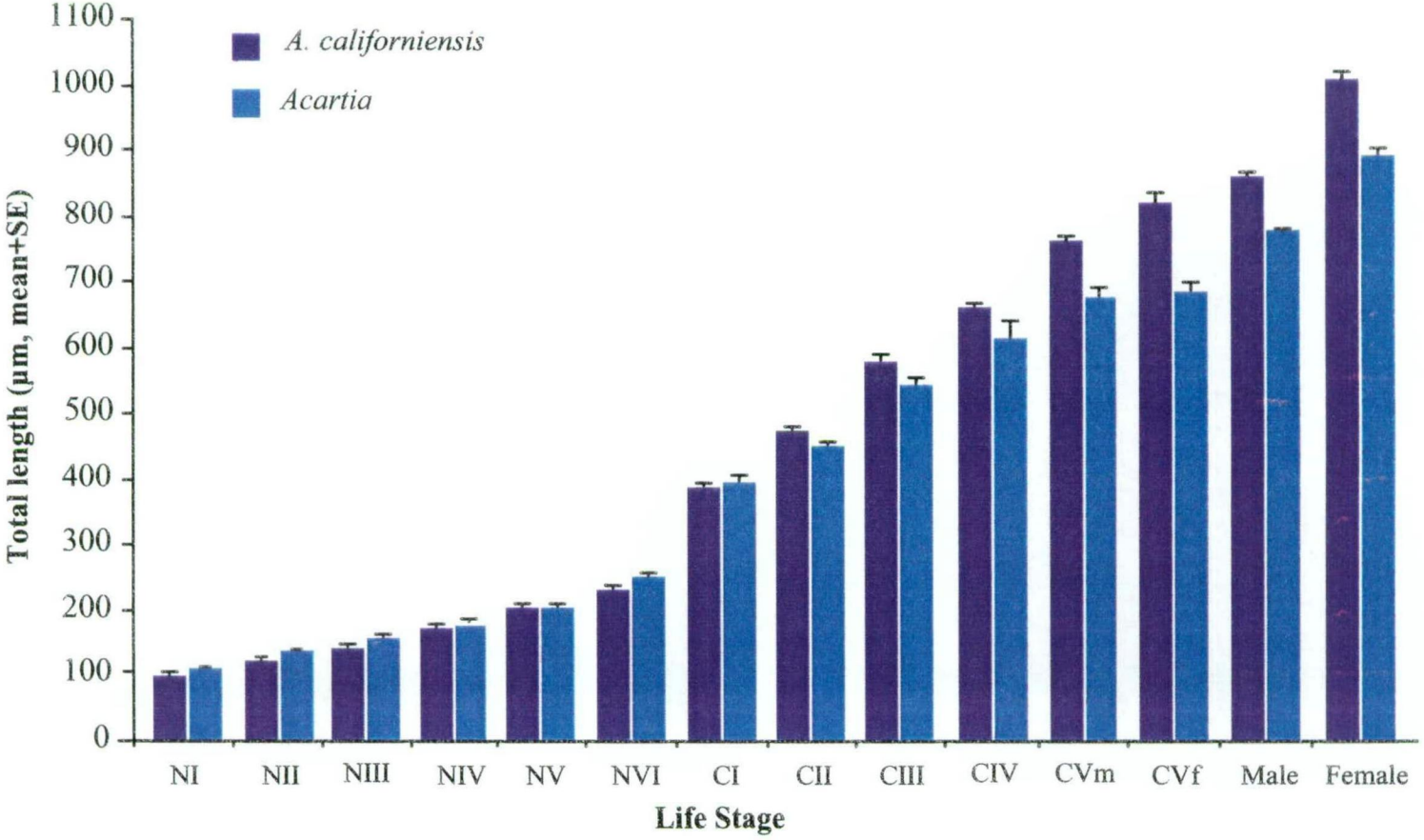


Figure 4.4.1: Comparison of the total length (measured, in µm, from the rostrum to the end of the last urosomal segment excluding the feruncular setae; mean + standard error) of *Acartia*: the Northern Territory species and *A. californiensis* (Trujillo-Oritz, 1986). The six nauplius stages are denoted NI through NVI, the sexually immature copepodid stages CI through CV. Sexual dimorphism evident at stage CV is indicated by the suffices m and f, with sexually mature CVI recorded as Male and Female.

4.4.2 Culture of *Acartia*

Populations of the tropical *Acartia*, although able to tolerate a wide range of temperature and salinity conditions, exhibits highest growth over a narrow range of salinity around 35 ‰. *Acartia* cultures perform best when presented mixed algal diet comprising *Isochrysis, Tetraselmis* and *Rhodomonas* species at densities of $1x10^4$ cells mL^{-1} maintained at 35 ‰ and 29-32 °C. Light levels and culture volume also influence *Acartia* culture success. Moderate to high light intensities combined with culture volumes greater than 500 mL produce more consistent densities than cultures maintained under low light conditions in volumes less than 500 mL.

The inoculation of experimental cultures throughout the investigation involved the use of copepodids. A short-term trial was conducted to identify the optimal initial stocking density to maximise consistency of results using the minimal inoculation density. An inoculation equivalent to 80 *Acartia* L^{-1} was shown to yield cultures of similar productivity to cultures inoculated at the higher densities equivalent to 160 and 320 *Acartia* L^{-1}. Trials subsequently conducted at DAC identified a copepodid stocking density of 60 *Acartia* L^{-1} to yield the most nauplii achieving densities of 1 mL^{-1} (Jerome Bosmans, DAC, *pers. comm.*).

Variation between replicates within stocking density treatment levels was high possibly as a result of variability of the inoculum composition in terms of the copepodid stages used. Increased replication may reduce the standard error, however a method by which inoculum composition can be rendered more homogenous needs to be developed. The use of eggs has been ruled out as a result of inconsistency in availability, difficulty in separating from culture detritus and surfaces, and variable hatch rate (Appendix C2).

Nauplii cannot be handled individually as any form of manipulation has resulted in a failure to develop through to copepodids and onto maturity. It is thought that damage to setae affects moulting ability (Glenn Schipp, DAC, *pers. comm.*; Gale Semmens, QDPI , *pers. comm.*). All trials conducted with the Northern Territory *Acartia* were subsequently inoculated with copepodids derived from cultures of known age, targeting later developmental stages CIV to CVI.

The response of the Northern Territory species of *Acartia* to temperature over the range of 23 to 35 °C was minimal in comparison to the response to salinities over the range of 5 to 45 ‰. No work has been published for tropical species assessing the response of *Acartia* to various combinations of temperature and salinity under controlled conditions.

Work conducted by Pagano & Saint-Jean (1989, 1993, 1994), Hopcroft et al. (1998) and Roff et al. (1995) in tropical coastal waters, and that of Ohno &

Okamura (1988), Ohno et al. (1990), Doi et al. (1994 a,b,c), Singhagraiwan et al. (1994) and Sunyoto et al. (1995) in tropical mariculture ponds constitute the basis of production knowledge for tropical *Acartia* species. No attempt to control temperature and salinity was made as the investigators were monitoring extensive systems. Seasonal influences driven largely by salinity, and to a lesser extent temperature, resulted in a succession of *Acartia* species dominance in a coastal seawater pond in Thailand. Ohno et al. (1996) observed *A. sinjiensis* to be present throughout the year with *A. erythaea* and *A. pacifica* occurring in high numbers between March and April at the end of the warm Wet Season.

Salinity was observed to exert a strong influence on Northern Territory *Acartia* culture productivity, temperature effects being almost non-existent in comparison. Productivity was significantly greater at 35 ‰ across all temperatures, with eggs more numerous at lower temperatures most probably as a result of slowed development. Pagano & Saint-Jean (1994) reported temperature and salinity to have no effect on fecundity of tropical *Acartia clausi*. Recognising the strong influence of salinity of the population of the Northern Territory *Acartia*, it is reasonable to suggest that the species may be a coastal not brackish water species. The narrow salinity range supporting *Acartia* productivity also explains the observed seasonality of the species in the coastal lagoon from which it was collected.

The coastal lagoon Vesty's Lake from which the original *Acartia* population was sourced, was monitored on a monthly basis for a twelve month period by DAC. Qualitative assessment of the environmental data and zooplankton species composition suggests that the presence of *Acartia* is seasonal, salinity being the dominant influence. During the tropical Dry Season (May through October) Vesty's Lake experiences a temperature range from approximately 20 to 30 °C coincident with an increase in salinity from near fresh to 37 ‰ as the influence of Wet Season runoff diminishes. *Acartia* is present in significant numbers from the middle of the Dry Season until the salinity of the coastal lagoon is lowered by the Wet Season rains, usually in late October (Jerome Bosmans, DAC, *pers. comm.*). The cycle is suggestive of the presence of a single species of *Acartia* in the Darwin coastal lagoon.

Investigation into the influence of algal diet species composition on *Acartia* production was limited by the impending relocation of the DAC research facility. Preliminary investigations indicated that the dinoflagellate *Heterocapsa neie* would be beneficial to long term culture success (Appendix C6) were supported by findings in Queensland. Similarly the value of the Northern Territory *Rhodomonas* species was recognised both in my work and that conducted by Gale Semmens in Queensland. Similar success had been experienced when using *Rhodomonas* species with Northern Hemisphere *Acartia* species (Støttrup et al., 1986; Berggreen et al., 1988; Jónasdóttir, 1994). In contrast to published work by Williams &

Parrish (1978) the prymnesiophyte *Isochrysis* is a valuable dietary component for *Acartia.*

An extended trial assessing the influence of diet algal species composition conducted in 10L cultures experiencing daily algal additions and a culture medium exchange every eight days confirmed the beneficial nature of *Isochrysis* to *Acartia* culture growth and the potential of *Rhodomonas* as culture diet (Appendix C7). Treatment diets containing *Rhodomonas* performed best, and worked well when presented in conjunction with *Isochrysis.*

Minimum effective cell composition for *Isochrysis* may be in the vicinity of 70% of total cell density on the basis of results from algal species diet trial (Section 4.3.2.4). The low culture densities recorded (less than 150 *Acartia* L^{-1} after six days, Figure 4.3.7) is significantly less than the average density for *Acartia* cultures maintained on mixed diets at lower cell densities for the same period (approximately 1300 *Acartia* L^{-1} after six days, Figure C6.1). The primary difference between the two trials was the use of cell carbon concentrations and inclusion of *Heterocapsa* and *Rhodomonas* in the algal cell carbon assessment. The results obtained from the short term trials suggest that *Isochrysis*, *Heterocapsa* and *Rhodomonas* represent high quality food items for the Northern Territory *Acartia.* Semmens et al. (1999) confirmed the value of *Heterocapsa neie* and *Rhodomonas* sp. as diets for the Northern Territory *Acartia* species, naupliar production one third to two times that achieved with *Isochrysis galbana* and *Tetraselmis* sp. presented at $1.5x10^4$ cells mL^{-1}.

Algal species would appear to exert a greater influence on *Acartia* culture densities than cell density as greatest *Acartia* densities were obtained in both trials from diets containing similar concentrations of algal cell carbon in the range from 100 to 5000 µgC L^{-1}. Microalgae cell density trials identified lower concentrations (2-$9x10^3$ cells mL^{-1}) to be sufficient to maintain healthy culture populations of *Acartia* (Appendix C6). Cultures maintained at lower carbon concentrations were most productive, which may be a reflection of the naturally lower carbon concentrations evident in tropical marine waters, and the reduced influence of algal activity on water quality at lower algal cell densities.

Ohno & Okamura (1988) found that in extensive pond culture of *A. tsuensis* productivity of copepod cultures were closely related to chlorophyll a <10µg L^{-1} concentration and hence to fertilisation rate, however excessively high levels of chlorophyll a resulted in a decline in the production of copepodids and adults.

Culture conditions in terms of culture volume, and the quality of light illuminating *Acartia* cultures strongly influenced culture success. Cultures maintained under artificial light conditions produced ten times more *Acartia* than cultures maintained under natural sunlight conditions (240 ±74 individuals compared with 21.3 ±6.5 individuals; Appendix C3). Marshall (1973) reported full sunlight to be lethal to

the calanoid *Calanus finmarchicus* due to increased oxygen consumption and direct damage to the animals. The high light intensity combined with the elevated temperatures and high pH levels may well have been to the detriment of the outside copepod cultures maintained under natural light conditions. The cultures exposed to natural sunlight experienced elevated temperature, pH and dissolved oxygen levels (33 °C, pH 9.7 and 11.6 mgO_2 L^{-1} compared to 30 °C, pH 8.0 and 7.2 mgO_2 L^{-1} Appendix C3). The trial conducted in 500 mL cultures identified artificial light of 500 lux to be as effective ambient shaded light conditions corresponding to 30,000 lux. A large variation in culture densities associated with the results may have masked greater population growth achieved under natural low light levels. However from the perspective of maintaining cultures under artificial and controlled conditions, the knowledge that supplemental lighting to 500 lux offers a significant advantage to *Acartia* culture is of importance.

It is evident that small volume cultures do not support continued *Acartia* culture productivity with 500 mL the smallest volume supporting reasonable increases in population density.

A comparison of culture density in the preliminary trial (Appendix C3) in which 150 mL cultures yielded a maximum density of 200 *Acartia* L^{-1} and 600 egg L^{-1} (total of 800 life stages L^{-1}) compared with an average culture density of 700 individuals L^{-1} and 400 eggs L^{-1} (total of 1100 life stages L^{-1}) from 500 mL cultures exemplifies the value of larger culture volumes. The greater density from the larger volume cultures maybe in part due to improved hatch rates and reduced cannibalism, both as a result of the greater buffering capacity of the larger volume of culture medium.

The *Acartia* nauplii densities recorded in this investigation (maximum 1143 L^{-1}) are greater than those reported from Thailand where populations of *A. sinjiensis* were maintained at 33-35 ‰ and 29.7 °C in outdoor ponds (7 m^3 and 30 m^3). Doi et al. (1994) reported maximum nauplius densities of 577 L^{-1} by day eight which also experienced a decline in association with an increase in copepodid numbers. The observed decline in naupliar numbers may be attributed to a combination of metamorphosis and cannibalism by developing copepodids on early stage nauplii (Ohno & Okamura, 1988; Doi et al., 1994b; Sunyoto et al., 1995).

Recognising similarities between the culture conditions under which *Acartia* exhibits highest productivity and those favouring productivity in the temperate calanoid *Gladioferans imparipes*, the potential exists to apply the system developed by Payne & Rippingale (2001) for the *G. imparipes* to the mass culture of *Acartia*. The systems developed for *G. imparipes* extends on the system developed by Schipp et al. (1999) incorporating a specialised nauplii collection tank, and a recirculation system for the reconditioning of culture water for reuse.

4.4.3 *Acartia* and golden snapper larvae

The true potential of *Acartia* as a live food was not demonstrated by the small scale trials conducted with golden snapper larvae. The relative lack of success achieved with these trials may be largely attributable to larval quality as all concurrent trials in larger systems experienced similarly high larval mortalities.

The emaciated state of the golden snapper larvae suggests feeding had not been initiated since their mouths opened late at 2 dph. Live food was not presented until 8 pm on the second day after hatching, possibly coinciding with the commencement of exogenous feeding. Similar poor survival rates were obtained with larvae presented food 6 hours earlier, which would imply that food deprivation was not the sole factor affecting survival of the golden snapper larvae. It is recognised that suitable artificial diets are unlikely to be available in the near future, so there is presently a need to develop a system for the reliable production of an alternative live food source for marine fish larvae (Fujita, 1973; Støttrup et al., 1986; Person-Le Ruyet et al., 1993; Sorgeloos et al., 1995).

The apparently healthier appearance of the larvae in the alternative aquaria which lacked a white centre pipe, and received a different form of aeration is a point worthy of further investigation. Possible benefits to golden snapper larvae afforded by the removal of the centre pipe may have related to improved visibility of prey, improved water flow and or aeration. Future trials including aquarium design as a factor were proposed but did not eventuate as a consequence of unsuccessful golden snapper broodstock spawning and winding down of research at the DAC due to the impending relocation of the facility.

Larval rearing of golden snapper has not been successful under intensive conditions in volumes less than 5 m^3, even with the use of *Acartia* blooms (Glenn Schipp, DAC, *pers. comm.*). The sensitive nature of the golden snapper larvae may be attributed to the prominent cupulae of the neuromasts (the sensory receptor unit which detects hydrodynamic displacements by prey, predators or social partners) located on their lateral line (Bagarinao, 1986; Doi & Singhagraiwan, 1993).

Golden snapper reared in 40 m^3 tanks at DAC have been observed to seek out *Acartia* nauplii present at densities less than 0.1 nauplius mL^{-1} in preference to the smaller rotifers *Brachionus rotundiformus* present at densities of 5 rotifers mL^{-1}. Doi et al. (1997c) observed similar behaviour in red snapper with *Lutjanus argentimaculatus* larvae selectively feeding on *Acartia tseunsis* nauplii present at densities of less than 1 mL^{-1}.

The success achieved in the rearing of tropical golden snapper larvae using semi-extensive larviculture practices with *Acartia* as the primary live food available at the time of first-feeding both in the Northern Territory and South East Asia

suggests it has potential and further investigation into the development of further culture techniques is warranted.

4.4.4 Summary of findings for *Acartia*

The north Australian species of *Acartia* demonstrates many characteristics documented in the literature and exhibits considerable potential as a live food for larviculture. In terms of the objectives identified in the introduction:

- *Acartia* exhibits a typical calanoid life cycle with twelve free-swimming stages ranging in size from 107 µm to 889 µm in length with a range in width from 63 µm to 333 µm.
- Initial separation of stages based on significant dimensional differences is able to be further refined using gross morphological features including segmentation and morphology of antennules, fifth periopods and urosome (Table 4.4.1).
- *Acartia* exhibits significant live food potential in terms of food particle size possessing at least four nauplius stages (NI through NIV) with widths less than 100 µm (Table 4.3.1).
- *Acartia* exhibits a mean generation time in the order of 7 to 8 days at 29 °C, naupliar stages being present for three of these.
- Culture populations maintained at 35 ‰ and 29-32 °C and fed a mixed algal diet comprising Isochrysis, Rhodomonas and Tetraselmis at a cell density of 1x104 cells mL^{-1} under high intensity artificial light achieve densities of 1,200 *Acartia* L^{-1}.
- Environmental and food conditions exert significant influences on the population as a whole. The closer to optimal the culture conditions, the greater the population density sustained and the more consistent are the numbers of nauplii present in the population.
- *Acartia* may improve the rate of survival of barramundi larvae (Appendix A9) and it has been recognised as an essential component in the diet of first feeding golden snapper larvae.

Chapter 5
Final Discussion

5.1 Australian copepods as live food organisms

The preceding chapters have addressed the life cycle, culture characteristics, and the interactions with fish larvae displayed by the three Australian copepods: *Tisbe* sp., *Apocyclops dengizicus* and *Acartia* sp.. The information collected herein has enabled the potential of the three copepod species to be assessed as live food organisms in light of the criteria identified in the introduction to the thesis.

All three Australian copepod species possess stages in their life cycles of appropriate dimensions which are: (a) available to the larvae in their environment, (b) stable until eaten, (c) visible and attractive, (d) palatable, and (e) not aggressive toward finfish larvae. Furthermore, the culture of the copepods facilitates the provision of pure, parasite- and pathogen-free live food organisms which are procurable and reproducible.

All three Australian copepod species were able to be maintained in culture achieving densities similar to those reported for related species from the Northern Hemisphere. Further work based on the knowledge gathered during the completion of the trials conducted is required to develop reliable cultures for use in large-scale commercial systems.

Due to time limitations, the nutritional composition of the three species was not specifically addressed, however, published information suggests that copepods in general do exhibit appropriate nutritional composition for growth and survival of finfish species. Preliminary work conducted by myself at the CSIRO marine laboratories indicated that *Tisbe* and *Apocyclops* do possess significant levels of HUFA, especially AA, EPA and DHA, comparable to published information (Appendix E; Tables E1.3.1 and E1.3.2).

No attempt was made to investigate the influence of diet on biochemical composition of the copepods. It has already been documented that the diet of temperate copepods can exert some influence over fatty acid compositions, with protein levels essentially unaffected. Reduced variation in chemical composition of tropical species has been observed (Lokman, 1993; Pagano & Saint-Jean, 1993; Shansudin et al., 1997). Toledo et al. (1999) found *Acartia* to have 24% DHA with a DHA: EPA of 2.6 compared with 13% and 1.4 for *Pseudodiaptomus*. Ederington et al. (1995) reported that lipid content of *Acartia tonsa* varied depending on diet, with the food source of prey ciliates also affecting the final lipid composition of *A. tonsa*. The best food however is microalgae or herbivorous ciliates. Lipids of bacterial origin (as indicated by the odd number of carbon atoms) are present in copepods fed ciliates indicating trophic transfer adding support to the suggestion that copepods form a significant link between the microbial food chain and traditional food webs (Roff et al., 1995; Hopcroft & Roff, 1998). The importance of microzooplankton in the diet of *Acartia tonsa* is further quantified by Gifford &

Dagg (1988) who found microzooplankton accounted for up to 41% of the total dietary carbon ingested.

The economics of copepod production remain a moot issue; copepods are relatively expensive to maintain in intensive systems. To obtain 500,000 rotifers in culture at the present time requires 20 L volume at an average cost of Aus$32 based on current production techniques adopted at the DAC (Table 5.1.2). To obtain 500,000 *Acartia* nauplii under intensive conditions maintaining *Tetraselmis, Rhodomonas* and *Isochrysis* at a total daily density of $2x10^5$ cells mL^{-1} (Schipp et al., 1999) would require a 1 m^3 conical tank at a cost of Aus$325 (Table 5.1.2). The production of *Acartia* is approximately ten times the cost of rotifers without considering the relative costs differences in infrastructure support for the maintenance of live food stock cultures. The use of extensive greenwater systems, or the semi-extensive larval rearing system using 40 m^3 tanks inoculated with culture copepods stocks and maintained at low densities (Schipp et al., 1999), would significantly reduce the costs of *Acartia* production largely attributed to the labour required to clean and feed the copepod cultures and maintain microalgae.

Table 5.1: Comparative component costs associated with the intensive production of 500,000 of both rotifers and *Acartia* at the Darwin Aquaculture Centre (assuming support systems and stock cultures have been established).

	rotifers		***Acartia***	
	Item	Cost	Item	Cost
Culture vessel	20 L		1000 L	
Water	40 L	negligible	1000 L	negligible
Stocking density	50 rotifers mL^{-1}		50 adult *Acartia* L^{-1}	
Lead time	2-3 days		3 days	
Algae	1.5 mL paste[#]	$0.75	30 L culture[##]	$270
Labour	1 hour	$27.40	2 hours	$54.80
- cleaning	- 45 min		- 75 min	
- feeding	- 5 min		- 30 min	
- counting	- 10 min		- 15 min	
Total		$32.15		$324.80

[#] 1 L of algae paste costs Aus$50. [##] calculated assuming 10 hours per week at Aus$27.40 hr^{-1} for a technician to inoculate and maintain 20 L carboys of algae.

If finfish larvae cannot survive on any other available live food, then cost is not a primary consideration. However, if the use if copepods is under review where rotifers and *Artemia* result in acceptable survival and growth rates of larvae of commercial finfish species, then the additional costs incurred in maintaining

copepods need to be taken into consideration. Where copepods represent a viable alternative to *Artemia* the relative cost of copepod production is less prohibitive, as is presently the situation with *Apocyclops royi* in Taiwan (Cheng et al., 2001; Su et al., 2001).

The objective of copepod culture systems is not necessarily to maximise the growth of the copepods themselves, but rather to establish a production system where total production is both high and stable (Støttrup et al., 1986). Maximum productivity is not determined solely in a biological sense. The economic climate of today necessitates consideration of productivity in terms of dollars input and dollars returned. The labour and material inputs required to achieve zooplankton exhibiting optimal nutritional composition for fish larvae must be considered in terms of the net benefit derived from reduced larval mortality.

5.2 Copepod life cycles and demographics

From an applied aquaculture perspective it is important that stages in the life cycles of copepods be easily identified to assist with assessment of copepod culture health, population growth and harvest.

Twelve life stages were identified in the life cycles of the Australian species of *Tisbe*, *Apocyclops* and *Acartia*; including six nauplius and six copepodid stages, the sixth copepodid stage corresponding to the sexually mature adult. The eggs of all three copepods were observed to be approximately spherical with diameters ranging from 75 µm to 90 µm.

The incremental development exhibited by the copepod species resulted in the size of copepod stages being a useful diagnostic feature. Distinctive morphological characteristics are also specific to each developmental stage. These features may be used to confirm initial separation of stages based on length and width data. Environmental conditions (including temperature, salinity and food availability) are known to influence size of stages (Klein Breteler et al., 1990; Milou, 1996). However, cultures grown for use in aquaculture would be maintained under consistent, near optimal, conditions rendering relative size a useful diagnostic feature. Published information suggests that size generally decreases with extended periods in culture, however there is a natural lower limit (Klein Breteler et al., 1990; Ohno et al., 1990).

The observations made herein for the Australian species conform with those reported for Northern Hemisphere congeners of the three copepods investigated and represents benchmark documentation of morphological details for Australian species of *Tisbe*, *Apocyclops* and *Acartia*.

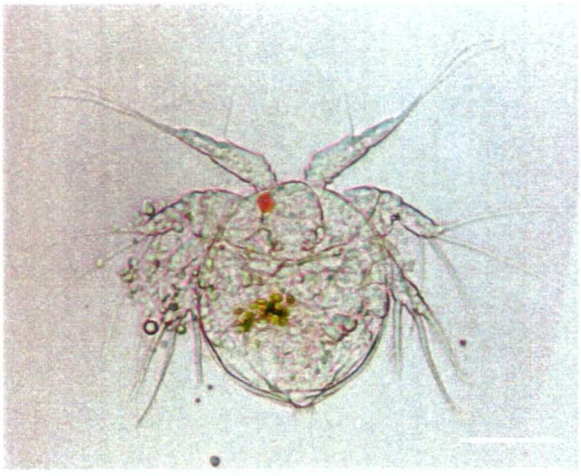
Direct species comparisons were not possible for *Tisbe* and *Acartia* due to the lack of information on Australian representatives of the two genera complicated by the

complexity of their taxonomy at the species level (Greenwood, 1972; Dahms & Schminke, 1995). Comparison of *Apocyclops dengizicus* with published information has confirmed considerable similarity between geographically distinct populations of the species.

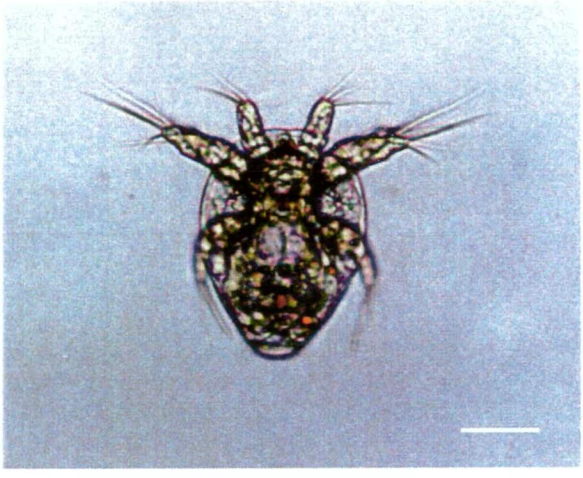
Similar morphological changes were observed across the three species with respect to naupliar development, with the posterior region of the nauplii becoming more elongate and structurally complex, and with each stage being easily recognisable under a dissecting microscope. The nauplii of all three species were distinguishable primarily on the basis of body shape (Figure 5.2.1) with the length to width ratios highlighting the more spherical appearance of *Tisbe* (L:W 1.2 to 1.4; Table 2.3.1), the more pyriform (i.e. oval/teardrop) shape of *Apocyclops* (1.4 to 1.7; Table 3.3.1), and the almost rectangular appearance of *Acartia* (1.7 to 5.1; Table 4.4.1).

The progressive development exhibited by the copepodids of the three species was also broadly similar, with species easily distinguishable on the basis of body shape, antennule morphology, and urosome structure and armature. Sexual dimorphism was clearly evident in all three species by copepodid stage five. The gender of sexually mature copepodids was also easily determined under the dissecting microscope.

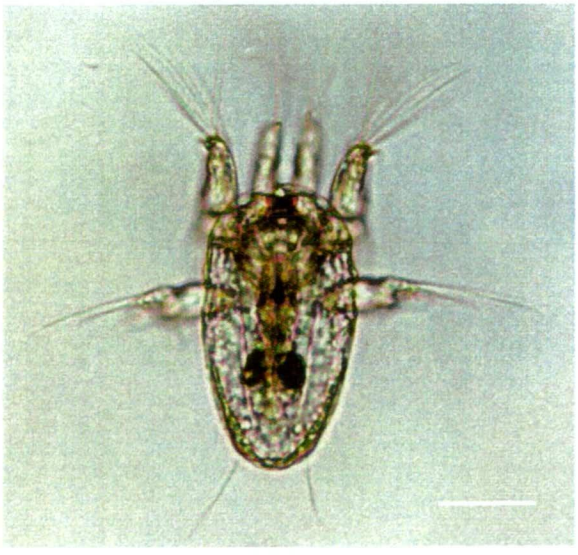
Prey size is a fundamental criterion determining the suitability of copepod candidates as live food (Shirota, 1970; De Silva & Anderson, 1985; Tucker, 1992; Ghan & Sprules, 1993; Utne-Palm, 2000). Marine finfish larvae generally possess mouths corresponding to a maximum prey size in the order of 100 µm at the commencement of exogenous feeding (Nellen, 1985; Watanabe & Kiron 1994; Iglesias et al., 1994). Of the three copepod candidates investigated here, only *Tisbe* possesses life stages with a total length less than 100 µm (Figures 5.2.2). However it is prey width, not necessarily length, which is the more important dimensional consideration for ingestion (Shirota, 1970; Ghan & Sprules, 1993; Fernández-Diaz et al., 1994).



(a)



(b)



(c)

Figure 5.2.1: Comparison of naupliar morphologies at stage NI for the three species investigated: a) *Tisbe* sp., b) *Apocyclops dengizicus*, and c) *Acartia* sp. Scale bars are 50 µm.

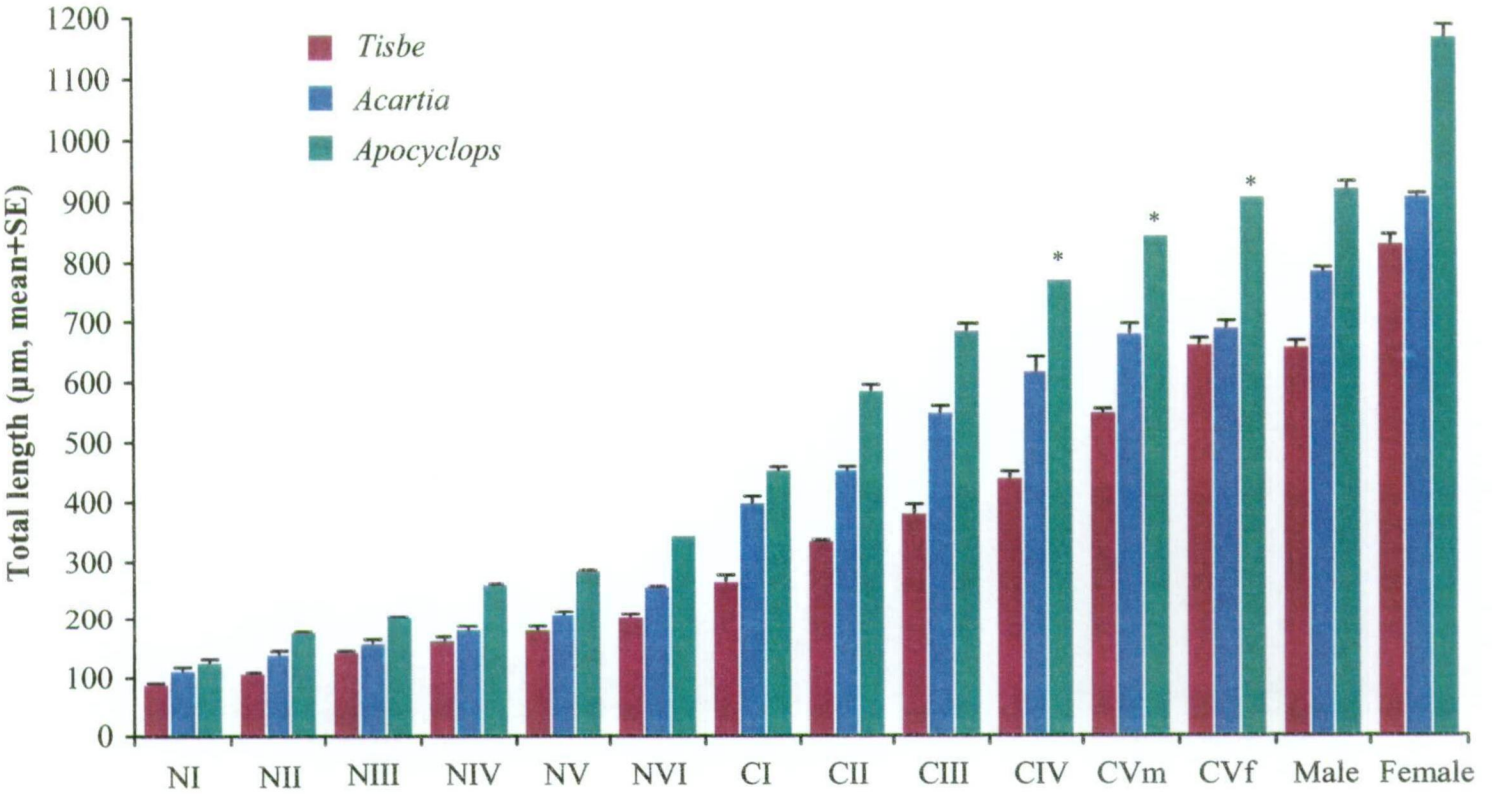


Figure 5.2.2: Lengths of the life stages of the Australian species of *Tisbe*, *Apocyclops* and *Acartia* (measured from the rostrum to the end of the last urosomal segment excluding the feruncular seate, measurements in µm, mean + standard error). NI through NVI indicate nauplius stages, CI through CV the immature copepodid stages. Sexual dimorphism evident at CV is inidicated by the suffices m and f, with Male and Female denoting the sexually mature CVI. *indicates the use of an estimated value.

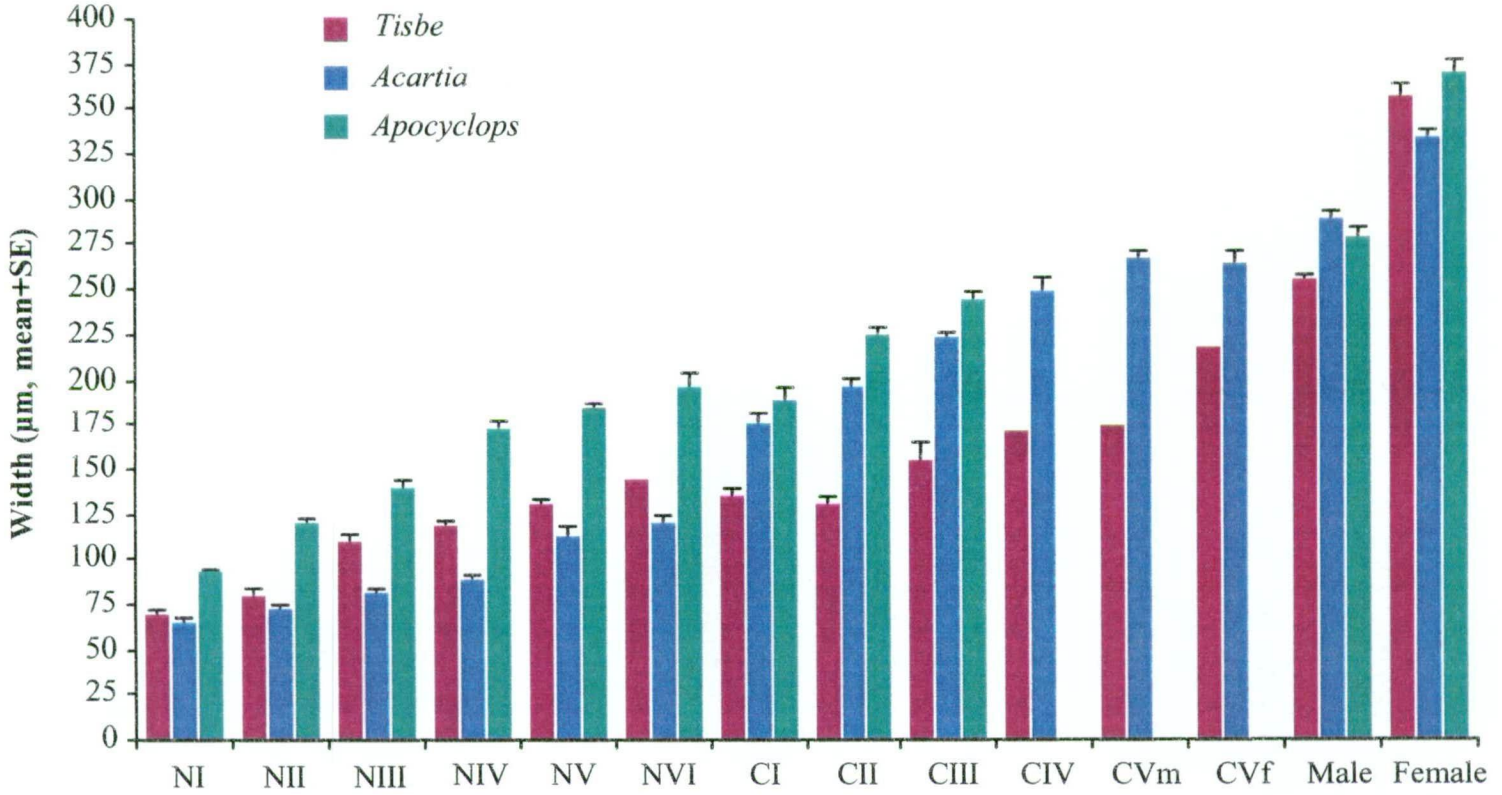


Figure 5.2.3: Widths of the life stage of the Australian species of *Tisbe*, *Apocyclops* and *Acartia* (measured at the widest point of the cephalosome, measured in µm, mean + standard error). NI through NVI indicate nauplius stages, CI through CV the immature copepodid stages. Sexual dimorphism evident at CV is inidicated by the suffices m and f, with Male and Female denoting the sexually mature CVI.

All three copepod species investigated here possess life stages with widths less than 100 µm (Figure 5.2.3) indicating potential suitability as live food organisms for marine finfish larvae which target prey items of 100 µm in size compared with the larger rotifers (50 to 200 µm) and *Artemia* (420 to 8000 µm) (Bromage & Roberts, 1995). The theoretical suitability of the three copepods as live foods for larviculture based on size alone is confirmed by the observed consumption of *Tisbe*, *Apocyclops* and *Acartia* nauplii by larvae of flounder, barramundi and golden snapper respectively.

The distinct size ranges displayed by each of the developmental stages of the copepod species also presents the opportunity to offer various size fractions of copepods to fish larvae of differing ages, and facilitates the isolation of the desired stages for the inoculation of trials.

In addition, the similarity of shape and mode of movement exhibited by the six nauplius stages within each species presents the opportunity to provide larvae with live food items of increasing size while optimising nutritional return per unit of energy expenditure (Cunha & Planas, 1995). Coincident with this is the added benefit that larvae do not experience a period of reduced feeding as the confounding effects associated with the introduction of novel prey, as witnessed with the introduction of *Artemia* after the commencement of feeding initiated on rotifers (Meyer, 1986; Cox & Pankhurst, 2000), is not evident. *Artemia* and rotifers exhibit significantly different modes of movement and colour, in addition to size disparity – factors which influence larval feeding (Utne-Palm, 1999; Utne-Palm, 2000).

The ability to distinguish reliably between the life stages of *Tisbe*, *Apocyclops* and *Acartia* is important to attain fine demographic data which would enable objective quantification of population growth and subsequent comparison of treatment effects. Distinguishing characteristics aid a more rapid determination of population composition, and quantification of population growth and health. The determination of specific developmental stages is fundamental to understanding the population dynamics and evaluating the production of a species (Yoon et al., 1998).

Similar rates of development were exhibited by the three copepod species at temperatures of approximately 30 °C. Males were observed to develop slightly faster than females in *Tisbe* and *Apocyclops*. It is likely that this trend would have also been apparent in *Acartia* if cultures of *Acartia* could have been maintained and monitored in the same rigorous manner as cultures of *Tisbe* and *Apocyclops*.

The six nauplius stages of the three copepods studied here each had a duration of approximately three days. Copepodid development times varied from three days for *Apocyclops* to four days for *Tisbe*.

The first three nauplius stages of *Tisbe* and *Acartia* fall within the desired size range of live food for first feeding larvae of marine finfish. The three day duration of nauplius development at approximately 30 °C translates to a need for continued production of nauplii in order to maintain a supply of suitable live food for developing larvae. Maintenance of copepod cultures at temperatures below 30 °C would achieve an extension of the nauplius duration (Sections 2.3.2.4 and 4.3.2.3) and thus increase the window during which they can be fed to finfish larvae.

The short generation times of tropical copepod species' is not conducive to detailed demographic studies based on monitoring of individuals. From a technical perspective and value to aquaculture, the most appropriate measure of population growth is likely to be based on the number of individuals produced. The final density in terms of the total number of copepods developing in culture has proven a good indicator of health of a population considering the interaction between the condition of adult members of the population and subsequent nauplius production. Sex ratios are higher at lower population densities (Hicks & Coull, 1983) and they decrease with the age of culture populations (Lazzarretto, 1994).

The study of demographics based on cohort analysis, as attempted with *Acartia*, was only partially successful because of characteristics of copepod population dynamics – that is difficulty in establishing well defined cohorts, continuous reproduction and short generation times (Kimmerer & McKinnon, 1987; Vijverberg, 1989; Saiz et al., 1997).

Determining the mean generation time (T), natural rate of intrinsic increase (r) and net rate of reproduction (R_o) requires tracking the development of an individual organism and its progeny. The resources and logistics of such an exercise precludes its use in trials conducted by a single researcher, adding support to the observation by Hart (1990) that short development times of tropical species makes it almost impossible to obtain precise demographic data. For temperate species, observations made at 12 hourly intervals were required to obtain detailed information on copepod species with a generation time of 40 days. The equivalent for tropical species with a six day generation time would be observations at 1.8 hourly intervals, which is logistically very difficult.

Determining the optimal culture conditions to ensure a reliable supply of copepods, the information obtained from a number of individual cultures is no better an indicator than that obtained from the response of multiple individuals in mass cultures. Subsequently assessment of environmental parameters on culture success was determined using small volume mass cultures to identify the optimal culture conditions in terms of temperature, salinity and diet.

5.3 The culture of copepods for use as live food

The three copepods, *Tisbe*, *Apocyclops* and *Acartia*, were successfully maintained in culture under a variety of conditions including aerated and non-aerated batch cultures, and small volume flow-through systems. The use of flow-through systems had the advantage of reducing any confounding effects attributable to the influence of environmental factors on the diet offered under the range of conditions investigated.

The three copepods all exhibited similar responses to temperature. The productivity of cultures as indicated by final population density were highest at a moderate temperature, declining either side of the optimum: 29 to 32 °C for the tropical species *Apocyclops* and *Acartia*, and 18 to 20 °C for the temperate *Tisbe*.

The influence of salinity on *Tisbe* and *Apocyclops* in terms of population growth varied over time, with both species exhibiting an ability to acclimate to immediate culture salinities. In contrast, *Acartia* displayed a strong negative response in terms of population growth to salinities outside a narrow range. *Tisbe* cultures proved most productive at 35 ‰ however population densities did not differ significantly between 25 and 40 ‰. Similarly, the optimal culture salinity of 20 ‰ identified for *Apocyclops* was associated with actively reproducing cultures over the range from 10 to 40 ‰. The optimal culture salinity for *Acartia* was identified between 30 and 40 ‰, however the steep decline in population growth rate at these two salinities compared to that achieved at 35‰ would suggest that the optimal salinity range is in the order of approximately 33 to 38 ‰. The observed differences in levels of tolerance to salinity fluctuations may be explained by the natural habitat of the copepods themselves: *Tisbe* predominates in coastal rock pools, *Apocyclops* predominates in brackish waters, and *Acartia* predominates in open coastal marine waters, although some species are common in estuaries.

The characteristics indicative of a species suited for aquaculture identified in the introduction to this thesis were: (a) rapid turnover, (b) tolerance of a wide range of environmental conditions, (c) simple dietary requirements and (d) tolerance of handling (Section 1.6). The three Australian copepods investigated here each demonstrated at least two of these characteristics.

Regardless of whether copepods feed opportunistically or selectively, their diets are diverse (Kleppel & Burkart, 1995). The majority of aquaculture facilities are well versed in microalgal culture techniques as a result of algal requirements by both live foods and some cultured finfish species. With this in mind, the majority of the diets tested were based on microalgae. Artificial diets were not tested with tropical species at elevated temperatures because bacterial blooms may result from overfeeding. The bacterial blooms have the undesirable effect of stripping culture water of oxygen and rendering culture systems putrid to the detriment of the copepod populations.

5.3.1 *Tisbe*

The euryhaline nature, tolerance of varying cultures conditions, and negative phototactic response demonstrated by the Australian species agree with information published for the Northern Hemisphere species of *Tisbe*. The temperate Australian *Tisbe* species is relatively easy to maintain as indicated by its persistence under a variety of conditions and tolerance of a high degree of handling. Individual ovigerous females, copepodids and nauplii were able to withstand wet screening and movement between cultures via pipetting of individuals.

The optimal culture conditions were identified as a temperature near 20 °C, with salinity in the range of 25 to 35 ‰ when fed a mixed diet of *Tetraselmis* and *Isochrysis* at a final cell density greater than 10^5 cells mL^{-1} (see Chapter 2) Aeration and water exchange were shown to be beneficial when population densities approached (or exceeded) 2,000 individuals L^{-1}.

Flow-through and recirculating systems could not be investigated in detail as means by which to increase culture population growth rate because my research was interrupted and relocated from Tasmania to the Northern Territory. However, preliminary work conducted using various substrates to increase surface area of culture media such as the use of 'bioballs' (plastic spheres with numerous fingers with a high surface area to volume ratio designed for use in biological filtration systems) are worthy of further investigation. Støttrup & Norsker (1997) developed a promising bioreactor in a closed system for the continuous culture of *Tisbe holothuriae* which focused on increasing the surface area to volume ratio using similar substrates. The bioreactor exhibited promise but required further work to maintain sufficient food for the harpacticoids contained within.

The use of *Tisbe* as a live food organism has received significant attention in the Northern Hemisphere; there also being evidence of its importance in tropical systems. Tropical red snapper larvae have been found to ingest copepodids of *Tisbe* sp. at 12 dph by Singhagraiwan & Doi (1993) comprising 24 to 71% of the copepodids ingested through to 23 dph.

5.3.2 *Apocyclops*

The tropical Australian *Apocyclops* also exhibited the desirable characteristics of tolerance of handling and proliferation over a wide range of environmental conditions. Its positive phototactic response to directed light acts as a useful tool in the concentration and harvesting of cultured populations.

The tolerance of *Apocyclops* to handling and changes in salinity were similar to those observed for *Tisbe*. Repetitive transfer of individual females from stock cultures, through rinses, into experimental units was tolerated well, with ovigerous females retaining egg sacs throughout the entire process. Individual copepodids

and nauplii were able to withstand wet screening and movement between cultures via pipetting of individuals.

The most productive *Apocyclops* populations were obtained from cultures maintained at temperatures of 29 to 32 °C with salinity in the range of 20 to 35 ‰ when fed a mixed algal diet of *Tetraselmis* and *Isochrysis* at a density of 10^5 cells mL^{-1} (see Chapter 3). Aeration in small cultures was shown to be detrimental to culture population growth, however, aerated stock cultures have been observed to have a more homogenous distribution of copepods, *A. dengizicus* tending to occupy the upper portion of non-aerated 80 L cultures.

The research documented here is the first comprehensive investigation into the culture characteristics of *Apocyclops dengizicus.*

5.3.3 *Acartia*

The tropical Australian species of *Acartia* investigated here tolerated only a low level of handling. The positive phototactic nature of *Acartia* was used to concentrate copepodids in a region of stock cultures. Inocula were removed with a volume of water rather than using the pipetting techniques used on the other two species.

Harvesting eggs of *Acartia* from cultures proved more difficult than what is documented in the literature. The nature of tropical environments fostering rapid development of bacteria may be the reason underlying the sticky nature of the eggs produced by females. Transfer of *Acartia* nauplii was also detrimental to their survival; any handling of individuals, or rough handling when screening individuals, resulted in their death. The selection of ovigerous *Acartia* from mixed populations also proved far more laborious and less rewarding than I experienced with *Tisbe* and *Apocyclops* as a consequence of the lack of external eggs sacs (*Acartia* broadcasts its eggs as opposed to retaining them in egg sacs) and inability to withstand pipetting.

In response to difficulties obtaining inocula, it was observed that an initial stocking density of 80 individuals L^{-1} produced similar *Acartia* densities within a six day period as those stocked with 160 and 320 *Acartia* L^{-1}.

The most productive cultures of *Acartia* recorded were those maintained at 35‰ at temperatures over the range 29 to 32°C when fed a mixed microalgal species diet based on *Rhodomonas* at 10^4 cells mL^{-1} yielding populations equivalent to 710 *Acartia* L^{-1} (see Chapter 4). The influence of aeration was not specifically investigated, with gentle aeration in stock cultures used to maintain a homogenous distribution of microalgal feeds and to prevent stratification.

Collaborative work conducted with staff at DAC achieved success in the mass cultivation of *Acartia* in 7 m^3 and 40 m^3 tanks using information obtained from the

small scale trials. These large-scale trials conducted using cultured *Acartia* inocula had the advantage of culture purity with no contamination from barnacle larvae, prawns or cnidarians, as experienced when using wild harvested zooplankton as inocula.

The considerable range in the levels of population growth I observed amongst the three species of copepods may be a reflection of the nature of the copepods themselves. *Apocyclops* is a brackish water species tolerant of a wide range of conditions and, being omnivorous, it makes the most of available conditions. *Tisbe* also a coastal species is tolerant of a wide range of environmental conditions and it also exhibits a varied diet. In contrast, *Acartia* is a more coastal species unaccustomed to environmental fluctuations and it appears extremely sensitive to any deterioration in water quality. The original population of *Acartia* was obtained from a coastal lagoon subject to tidal influences. Monitoring of water quality and *Acartia* abundance over time revealed that this calanoid disappeared as salinities dropped and reappeared when conditions became more favourable (see Section 4.4.2). As a consequence of the variable environmental conditions prevailing in the coastal lagoon, *Acartia* supplies were unpredictable. The possibility that the northern Australian species of *Acartia* might produce dormant eggs to weather the unfavourable conditions prompted staff at DAC to investigate the possibility of medium term storage of *Acartia* eggs. Inconclusive results were obtained prior to resources being redirected. However it is possible to store *Acartia* nauplii at 4 °C for a period of three days prior to feeding active nauplii to tropical finfish larvae (Jerome Bosmans, DAC, *pers. comm.*).

Acartia species have been propagated in tanks and ponds, their use as a food organism supported by several authors (Støttrup et al., 1986; Sunyoto et al., 1995; Takahashi & Ohno, 1996; Doi et al., 1997b).

The potential exists, especially with respect to temperate calanoid species, to obtain resting eggs similar to that achievable with rotifers (Hagiwara et al., 2001; Marcus & Murray 2001). The advantage afforded by a dormant stage able to be prompted back to life at will would provide a means by which to store copepods until the nauplii were required as live food.

5.4 The suitability of the three Australian copepods for use in larviculture

The most comprehensive investigation into the suitability of an Australian copepod species as a live food for larviculture was conducted using *Tisbe* and greenback flounder. The trials successfully confirmed larval acceptance of *Tisbe* as prey, with first feeding flounder larvae targeting nauplii in preference to rotifers. Flounder of 15 dph were observed to ingest ovigerous *Tisbe* 355 µm in width and 825 µm in total length.

Trials with *Apocyclops* and barramundi larvae confirmed that barramundi larvae do consume all life stages of the cyclopoid. Barramundi larvae of 7 dph are accomplished predators reducing the number of ovigerous *Apocyclops* 370 µm in width and 1160 µm in length. Concerns expressed by barramundi farmers regarding the predatory nature of the cyclopoid and their subsequent belief that the *Apocyclops* was the cause of poor larval survival observed in their larval rearing ponds were unfounded. However weak or moribund barramundi larvae are consumed by *Apocyclops*.

Trials conducted in small volumes (less than 4 L) with *Acartia* and golden snapper were unsuccessful in terms of larval feeding and survival rates achieved. However larval rearing trials comparing the performance of rotifer-fed and *Acartia*-fed golden snapper in 40 m^3 tanks did support the hypothesis that *Acartia* nauplii were essential to achieve high survival rates in larval lutjanids. A 40% survival to 21 dph (Schipp et al., 1999) was achieved using semi-extensive larval rearing methods involving the use of *Rhodomonas* as an algal food for *Acartia* in a 40 m^3 tanks stocked with golden snapper eggs. Previously golden snapper survival was nil when larvae were fed s-strain rotifers.

The finding of Shirota (1970) that fish species with faster development commenced life with larger mouths and therefore could commence exogenous feeding on larger copepod life stages earlier, was reflected in the observation that species with smaller mouths targeted copepod nauplii in preference to rotifers. The general trend of developing fish larvae to target prey of increasing size with progressive growth (Hunter 1981) was also substantiated to a degree.

The observed preference by finfish larvae for copepod nauplii may be due to the different locomotion pattern of nauplii compared to rotifers: zig-zag movements of copepod nauplii with subsequent pauses for floating, and secondly their smaller size as indicated by Van der Meeren (1991).

The value of *Tisbe* as a live food has also been recognised in the culture of striped trumpeter larvae in Tasmania (Battaglene et al., 2000) and *Tisbe* has been reported as a significant component in the gut contents of 12 dph tropical red snapper larvae (Doi & Singhagraiwan, 1993). Trials with the temperate Australian species of *Tisbe* to achieve productive high density cultures using the techniques outlined by Støttrup & Norsker (1997) would be worth conducting. The habit of the Southern Hemisphere species of *Tisbe* closely resembles that of its Northern Hemisphere counterparts. The completion of medium-term larval performance trials with southern finfish species would be possible using the technology developed for Northern Hemisphere congeners of *Tisbe*.

Apocyclops dengizicus demonstrates characteristics desirable in a live food organism; that is appropriate size range, successful stimulation of feeding response in fish larvae, non-threatening and readily attainable in a pure state from artificial

cultures. The nutritional composition of the copepod has not been specifically addressed in this study, however preliminary analyses conducted by the author at the CSIRO Marine Laboratories in Tasmania, indicated that the fatty acid profile of the cyclopoid exhibited significant levels of the fatty acids AA, EPA and DHA deemed essential to larval marine finfish survival (Appendix E).

The potential of *Apocyclops dengizicus* as a live food is exemplified by evidence from South East Asia where *A. royi* from Taiwan, an easily cultured and important secondary producer exhibiting potential as food source for fish larvae, has been used by commercial grouper hatcheries (Chang & Lei, 1993; Su et al., 1997; Hsu et al., 2001). Similarly *A. borneonsis* demonstrated potential as a replacement for *Artemia* in Malaysia (James & Al-Khars, 1984).

Apocyclops dengizicus is able to be cultured over a wide range of environmental conditions on a variety of diets including traditional microalgal species and heterotrophic food sources such as waste fish food. The more regulated the culture system, the more consistent the quantity of individuals available.

Apocyclops dengizicus possesses only one nauplius stage with a width less than 100 µm suitable for feeding to marine finfish larvae with mouths capable of accommodating feed particles in the order of 100 µm. Barramundi larvae, which possess large mouths of 0.2 to 0.25 mm wide at first feeding (Lim et al., 1985a), would have no difficulty in consuming prey of 200 µm in width which corresponds to all six nauplius stages of *A. dengizicus*. However, it would not be suitable as an alternative to rotifers as a first food organism for marine larvae with small mouths such as golden snapper, barramundi cod and coral trout.

The potential value of *Apocyclops* as an alternative to *Artemia* is supported by James & Al-Khars (1984) work with sobaity and *Apocyclops borneoensis*. Although no differences in larval performance was noted in early stage sobaity larvae fed *A. borneoensis* when compared to *Artemia*-fed sobaity, older sobaity larvae fed *A. borneoensis* exhibited superior performance to those fed *Artemia* alone. In this instance *A. borneoensis* represents a high quality replacement for the more expensive *Artemia*.

Liao et al. (2001) indicated that *Apocyclops royi* in Taiwan possessed the greatest HUFA content and highest DHA to EPA ratio of any *Artemia,* rotifer or copepod. Giant grouper which have proven difficult to feed using rotifers (150-250 µm) fed well on oyster trochophores (60 µm) and *A. royi* nauplii (100 µm) which are both smaller in size and possess similar HUFA content (Liao et al., 2001; Hsu et al., 2001).

Acartia, in contrast to *Tisbe* and *Apocyclops*, appears to be an essential component of some tropical finfish larval diets. However more work is required to perfect mass culture techniques to yield reliable and consistent quantities of *Acartia*. The

batch scale-up culture system developed at DAC, and of which I was a team member, (Schipp et al., 1999) provides a basis from which to develop techniques with increased rates of population growth. The combined fragility of both the calanoid copepod and lutjanid larvae in culture systems suggests that either semi-intensive or extensive systems are likely to yield the most cost-effective results. Recent trials conducted at DAC using larvae obtained from F_1 domesticated golden snapper broodstock confirmed the importance of copepods to larval survival. Golden snapper reared on rotifers alone exhibited 2.5% survival to weaning compared to 22.5% when fed copepods (Schipp et al., 2001).

The life cycle of the northern Australian species of *Acartia* is conducive to future use in the larviculture of difficult-to-rear, high value, tropical marine finfish species such as golden snapper, red snapper and barramundi cod.

The dimensions of the twelve life stages and approximate 9-12 hour duration of each nauplius stage are compatible with the requirements of tropical finfish larvae. Red snapper larvae have been observed to ingest *Acartia sinjiensis* nauplii (58 µm in width) at the commencement of feeding, while the ingestion of rotifers (117 µm lorica width) was delayed for three days (Doi et al., 1997b). Selectivity indices indicated that red snapper larvae preferentially targeted copepod nauplii over rotifers (Doi et al., 1997b).

Similarly, Toledo et al. (1999) observed that red-spotted grouper ingested two to six times more copepods than larvae fed rotifers, despite copepod densities being up to fifty times lower than rotifer densities in larval rearing tanks (between 0.2 and 0.8 copepods mL^{-1} compared with 5 to 10 rotifers mL^{-1}).

The size of the northern Australian species of *Acartia* compares favourably with that of *A. sinjiensis* from Thailand. The first and last nauplii stages of *A. sinjiensis* with mean body length and width of 101 x 51 µm and 248 x 123 µm respectively (Doi et al., 1997b) compared well with the 107 x 63 µm and 248 x 117 µm observed here for the northern Australian species.

Red-spotted grouper also exhibited a preference for *Acartia* species over other calanoid and cyclopoid nauplii. Early stage nauplii of *A. tsuensis* targeted in preference to *Pseudodiaptomus* and rotifers (*Brachionus rotundiformus*), even when the abundance of nauplii was as low as 100 L^{-1} (Doi et al., 1997a).

The research undertaken for this thesis provided information on which the semi-intensive large scale larval rearing trials undertaken at DAC were based The culture of *Acartia* using a green-water system achieved 40% survival of golden snapper to 21 days post-hatch (Schipp et al., 1999).

5.5 The future of the three Australian copepods as live food organisms for larviculture

The preceding sections have outlined the considerable potential of all three Australian copepod species for successful use as live food for marine finfish larvae. The life cycles documented here for *Tisbe*, *Apocyclops* and *Acartia* have identified suitable developmental stages for consumption by first feeding larvae of marine finfish species with small mouths. When larvae of greenback flounder and golden snapper were presented with a choice between rotifers and copepod nauplii, the first feeding larvae expressed a preference for copepod nauplii over rotifers. The developing larvae of greenback flounder, barramundi and golden snapper also demonstrated the ability to ingest all twelve life stages of all three copepods.

Northern Hemisphere experience, in conjunction with success in South East Asian countries with new marine aquaculture species candidates such as grouper and snapper, lend support to the potential of copepod species as live food (Kahan et al., 1981; Norsker & Støttrup, 1994; Doi et al., 1997 a,b,c; Ali et al., 1998; Liao et al., 2001; Hsu et al., 2001). Brown-marbled grouper (mouth size of 193 µm) and rabbit fish (mouth size of 168 µm) were reared successfully when early stage nauplii of *A. tseunsis* or another *Acartia* species were provided for the first few days prior to feeding rotifers (Doi et al., 1997; Ali et al., 1998). Doi et al. (1997a) also reared red-spotted grouper larvae in outdoor tanks with nauplii of *Pseudodiaptomus annandalei* and *A. tseunsis* and/or rotifers (*Brachionus rotundiformis*). Successful first feeding was observed with larvae targeting early stage nauplii and showed better survival and growth thereafter compared with red-spotted grouper larvae fed rotifers only, even though naupliar abundance was as low as 100 L^{-1}. Selective feeding ability started from day 4 with larvae preferring medium to large nauplii in preference to rotifers as they grew (Doi et al., 1997a).

Similar results have recently been obtained by Australian researchers supplementing rotifers with cultured copepods. The feeding of mixed diets containing copepods and rotifers has been associated with improved growth and survival for a number of finfish species including the Western Australian dhufish and pink snapper (Payne et al., 2001). Western Australian dhufish larvae presented a mixed diet (50% rotifer with 50% calanoid *Gladioferens imparipes* nauplii) yielded larvae of 11 mm in length nine days in advance of dhufish larvae fed solely rotifers. Copepod supplemented larvae also exhibited a 550% better larval survival rate. Similarly pink snapper larvae reared on rotifers only were significantly smaller and exhibited a slower rate of growth then those fed calanoid nauplii for six days of feeding prior to being fed rotifers (Payne et al., 2001).

The use of copepod nauplii is associated with a higher incidence of feeding as observed with greenback flounder larvae and *Tisbe* nauplii (Section 2.3.3). The incidence of feeding exhibited by red-spotted grouper larvae reached 100 % on day

four when nauplii were available, compared with day nine when rotifers were given alone (Doi et al., 1997a).

Examples illustrating the importance of copepods to the survival of finfish larvae are numerous, and the preliminary research conducted with the target copepod species investigated exhibit similar trends. These results are highly significant for aquaculture.

Australian aquaculture relies on the production of larvae or juvenile fishes (= seed-stock) from hatcheries, in contrast to some overseas ventures where juveniles are collected from the wild. The cost-effective production of seed-stock in an environmentally appropriate manner requires research into larval rearing and husbandry techniques to preserve both wild fish stocks, and the biological diversity of both teleost and zooplankton species.

Shortage of *Artemia* combined with its nutritional variability, large size and high cost (Ohno & Okamura, 1988; Kolkovski, 2000; De Wolf & Candreva, 2001) has provided impetus for the development of replacements. In Taiwan the use of *Apocyclops borneonesis* has been contemplated (James & Al-Khars, 1984; Hsu et al., 2001). The use of copepods and their stimulation/supplementation of larval digestion may further facilitate early weaning of finfish larvae onto artificial diets further reducing reliance on *Artemia* by aquaculture ventures.

Environmental considerations with respect to species biodiversity and gene pool diversity adds another element in support of the development of alternative foods for larviculture. The impact of non-native *Artemia* spp. and *Brachionus plicatilus* strains on the Australian environment are as yet undetermined. In light of caution and recognising the benefit in using endemic live foods species with local finfish species, efforts should be maintained in the development of culture techniques for local species of copepods, rotifers and brine shrimps (e.g. *Parartemia* species).

In Australia research on copepods as hatchery foods currently occurs in five main centres: Darwin, Brisbane, Cairns, Adelaide and Perth (McKinnon, 2000). Work with the calanoid *Gladioferans imparipes* has been long term and proven in the green water culture of a number of fish species. *Acartia* species are the subject of investigation in Cairns, Adelaide and Darwin, exhibiting value as a live food for the high value species barramundi cod, brown-marbled grouper, red-spotted grouper, red snapper and golden snapper in the tropics, and the temperate King George whiting.

Work completed with *Tisbe* has proven the species to be of value in the culture of striped trumpeter (Battaglene et al., 2000). As with *Apocyclops*, *Tisbe* was not proven to be an essential dietary component of marine finfish diets, however they may have value as a supplement, or substitute, for *Artemia*.

The majority of investigations concerning biochemical composition of copepod species have been conducted mainly on Northern Hemisphere or temperate marine or freshwater species with little known about the chemical composition of brackish and or tropical zooplankton. Addressing this, Pagano & Saint-Jean (1993) found the proximate biochemical composition of *Acartia clausi* in a tropical brackish lagoon remained stable throughout the year despite significant variation in environmental conditions.

The development of mass culture techniques is necessary to facilitate research and development of culture techniques for new mariculture species, and the identification of the elusive factors rendering copepods such a successful larval diet.

The challenge still remains of analytically and biologically unravelling those components in wild copepods which are responsible for their excellent dietary value for marine larval fish (Person Le Ruyet, 1989; Sorgeloos et al., 1995) before live food can be replaced by artificial diets in larviculture.

The use of copepods as live food may still prove expensive and less convenient than rotifers and *Artemia*, however there is a proven requirement by some species for smaller live foods, if not specific enzyme, amino acid and fatty acid composition which cannot be ignored. The three Australian copepod species investigated here – *Tisbe* sp., *Apocyclops dengizicus* and *Acartia* sp. – all exhibit traits rendering them worthy of further investigation as live foods for use in Australian larviculture.

Chapter 6
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Table 6.1: Details of individuals with whom personal communications have provided input to this thesis.

Individual	Association
Dr G. John Purser	Senior Lecturer, School of Aquaculture, University of Tasmania
Mr Glenn Schipp	Senior Finfish Research Officer, Fisheries Division, Northern Territory Department of Business Industry and Resource Development
Mrs Gail Semmens	Live Prey Research Unit, Queensland Department of Primary Industries, Northern Fisheries Centre, Cairns, Queensland, Australia
Mr Jeff Reid	Owner and operator of Barramundi Farmers NT, Darwin
Dr David McKinnon	Australian Institute of Marine Science, Townsville, Queensland, Australia
Dr Robert Campbell	Marine Research Scientist, Graduate School of Oceanography, University of Rhode Island, Rhode Island, USA

Appendix A
Tisbe

A1 Introduction

The following trials were conducted to obtain indicative information concerning the response of populations of the temperate Australian species of the genus *Tisbe* to prevailing salinity and diet conditions.

Casual observation of *Tisbe* species (referred to as *Tisbe* in the remainder of the Appendix) stock cultures over time revealed healthy copepod populations persisted over a wide range of salinities when the cultures experienced gradual changes in salinity.

In aquaculture facilities it is common practice to rinse rotifers in fresh water for one minute to eliminate ciliates from the live food prior to being introduced to larvae culture systems (Hoff & Snell, 1993). Similarly, certain fish species are often cultured at salinities and temperatures differing from those yielding greatest live food production.

The first trial was conducted to assess the tolerance of *Tisbe* cultures to sharp changes in salinity, ovigerous females were isolated and exposed to rapid salinity changes of up to 40 ‰ (A2).

The second trial conducted related to the effect of seawater type on copepod productivity (A3). As early as 1969, Barr used artificial saltwater to circumvent the issue of metabolite contamination and subsequent reduced copepod performance in culture. The use of artificial seawater also has the advantage of eliminating the need for salt water collection and storage.

The third trial addressed the use of food alternatives to mobile single celled microalgae such as artificial fish feeds and a benthic diatom (A4). The diatom *Nitzschia* has been tried in the culture of abalone (John Purser, University of Tasmania, *pers. comm.*) and as such was available for assessment in terms of usefulness in the culture of *Tisbe*.

Copepods within the genus *Tisbe* have been reported as exhibiting two primary modes of feeding: surface browsing and filtration (Hicks & Coull, 1983). The Tasmania species of *Tisbe* has been successfully reared on single cell motile microalgae, the thalli of *Ulva* sp. and bacteria (Marshall, 1993). The detrital, surface browsing feeding habits of *Tisbe* as reported by numerous authors (Guidi, 1984; Hicks & Coull, 1983) encouraged the assessment of additional food sources which may be found in a larval rearing environment such as excess artificial fish feeds.

A2 Salinity tolerance

The survival and reproductive success of ovigerous *Tisbe* was assessed for twelve salinities: 0, 10, 20, 25, 30, 35, 40, 50, 60, 70 and 80 ‰.

A2.1 Materials and methods

Treatment media were prepared for each of the salinities using a combination of deionised water, Aquasonic® Ocean Nature artificial sea salt and 0.45 µm filtered natural seawater originally at 30 ‰.

Duplicate 70 mL cylindrical clear plastic culture units were filled with 20 mL of treatment culture medium for each of the twelve salinities. To each was added 3 small pieces equating to approximately 500 µg of dried mussel meat obtained by drying *Mytilus planulatus* meat at 60 °C for 24 hours. The pieces of dried mussel meat exhibited the desirable characteristics of extended shelf life, ease of management and reduced tendency

to putrefy culture media in comparison to mussel juice puree obtained by macerating mussel meat in seawater.

One hundred and twenty ovigerous *Tisbe* were individually pipetted from the sides of a stock culture maintained at 35 ‰ between 18 and 20 °C on a mixed algal diet of *Tetraselmis* and *Isochrysis*. Five *Tisbe* females were placed into each of the twenty-four culture units which were subsequently placed on a shaded bench in a constant temperature room maintained between 18 and 20 °C.

All twenty-four vessels were monitored three times on the first day: 5 minutes, 2 hours and 6 hours post-inoculation, after which the vessels were monitored daily for the duration of the trials (5 days for Trial A and 14 days for Trial B). Observations made related to the: presence or absence of egg sacs; the general state of female health assessed in terms of vigor and egg sac size; and the survival of resultant nauplii.

A2.2 Results

Ovigerous *Tisbe* are found to tolerate and reproduce successfully over a wide range of salinities from 25 to 50 ‰ (Table A2.1) when transferred from 35 ‰. The maximum salinity increment tolerated by ovigerous *Tisbe* would appear to be in the order of 15 ‰.

A2.3 Discussion

In this trial *Tisbe* females were exposed to an immediate salinity change, in the extreme cases of 45 ‰ resulting in death. The tolerance exhibited may be of ecological value in that salinity changes tested are comparable to those in exposed shallow tidal pools. The euryhaline nature of the copepod is of value from a culture point of view where brackish water fishes may exhibit optimal feeding at 20 to 25 ‰.

The difference in response observed for salinities below 25 ‰ and those between 60 and 80 ‰ may be attributable to differences between the ovigerous *Tisbe* used as the inocula. It is possible that the *Tisbe* females used for the five day trial were older than those used to inoculate the 14-day trial. Older copepods are recognized as being less resilient to changing environmental conditions than younger females, the age of copepods also affecting egg sac quality hence naupliar survival rates (Hicks & Coull, 1983; Milou, 1993).

The extended range of salinities (up to 80 ‰) over which continued *Tisbe* production had been observed in stock cultures by myself were reached via gradual change over periods of days to weeks. The extended time frame over which the salinity increases occurred enabled the gradual acclimatisation of developing copepods in contrast to the rapid salinity fluctuation experienced by ovigerous *Tisbe* in the following trial.

Geographical differences in *Tisbe* population tolerances to different salinities has been noted by Milou (1993) which suggests long term acclimatisation of cultures for optimal production at different salinities may be possible. However the increase in space required to maintain cultures at different salinities is not necessary when life stages are able to tolerate 10 ‰ changes in salinity.

The feasibility of using freshwater rinsing to eliminate ciliates from copepod cultures is not a realistic option as ciliates tolerate freshwater immersion in excess of 5 minutes (John Purser, University of Tasmania, *pers. comm.*).

Table A2.1: A summary of the tolerance exhibited by ovigerous *Tisbe* females when transferred from 35 ‰ to various salinities.

Salinity	Trial A (5 day duration)	Trial B (14 day duration)
0 ‰	ceased moving immediately, dead	ceased moving immediately, dead
10 ‰	ceased moving within 60 seconds, dead	ceased moving within 60 seconds, dead
20 ‰	dead within 24 hours	dead within 24 hours
25 ‰	dead within 48 hours	numerous egg sacs, nauplii through late copepodids evident after two weeks
30 ‰	alive after 5 days, nauplii present	numerous egg sacs, nauplii through late copepodids evident after two weeks
35 ‰	alive after 5 days, nauplii present	numerous egg sacs, nauplii through late copepodids evident after two weeks
40 ‰	alive after 5 days, nauplii present	numerous egg sacs, nauplii through late copepodids evident after two weeks
45 ‰	alive after 5 days, nauplii present	numerous egg sacs, nauplii through late copepodids evident after two weeks
50 ‰	fine after 5 days, nauplii present	numerous egg sacs, nauplii through late copepodids evident after two weeks
60 ‰	dead within 48 hours	dead within 72 hours
70 ‰	dead within 48 hours	dead within 72 hours
80 ‰	Ceased moving within 5 minutes, dead	dead within 24 hours

A3 Artificial versus natural seawater

The influence of seawater composition (natural seawater versus artificial seawater) on *Tisbe* productivity was assessed at the five salinities: 25, 30, 35, 45 and 45 ‰.

A3.1 Materials and methods

Twenty litres of natural seawater collected off the north coast of Tasmania was placed in an air-conditioned control room to reduce to a volume of 14 L as a consequence of evaporation increasing the salinity to 50 ‰. Fourteen litres of artificial seawater were made up to 50 ‰ by adding 700 grams of Aquasonic® Ocean Nature marine salt to 14 L of freshly deionised water. The hypersaline preparations were subsequently filtered through a 0.45 µm Millipore® filter under vacuum prior to the addition of algae and dilution to the appropriate salinity using 0.45 µm filtered deionised water.

One litre volumes of culture media were prepared for each of the five salinities: 25, 30, 35, 45 and 45 ‰. using natural and artificial seawater in combination with deionised water. *Tetraselmis* and *Isochrysis* were added to each of the preparations to achieve final cell densities of $1x10^5$ and $6x10^4$ cells mL^{-1} respectively. Five replicate cylindrical clear plastic 200 mL culture units were filled with 150 mL for each of the five salinities prepared using artificial and natural seawater.

Individual ovigerous *Tisbe* were pipetted from the sides of stock cultures A (Section 2.2.2) maintained at approximately 18 °C and 30 ‰ fed a mixed diet of *Tetraselmis*, *Isochrysis* and crumbled salmon pellets. The female copepods were rinsed in 0.45 µm filtered natural seawater prior to 3 ovigerous *Tisbe* being randomly transferred to each individual culture unit.

The forty culture units were randomly distributed between two treatment blocks on shelves located in a constant temperature room maintained at 20 °C. No aeration was supplied due to the small volume of cultures to reduce possible confounding effects on salinity as a consequence of variable rates of evaporation.

Water and ambient temperatures were monitored daily for the nine-day duration of the trial. On the ninth day 40 mL of a 1:1 mixture of formalin and glycerol was added to the contents of each culture unit (to achieve a final 1:1:8 formalin, glycerol, seawater mix) to preserve the copepods until time was available to enumerate the various life stages.

The number of females, males, late copepodids (CIV and CV), early stage copepodids (CI-CIII), late nauplii (NIV-NVI), early nauplii (NI-NIII) and eggs were enumerated using an Olympus SZ40 stereo dissecting microscope.

All environmental parameter and *Tisbe* data collected was subject to Shapiro-Wilks test for normality and Bartlett's test for homogeneity of variance. Treatment effects in normal data exhibiting homogenous variance were determined by analysis of variance (ANOVA) and Scheffe's multiple means comparison tests. Data unable to be transformed to meet the assumptions of ANOVA were analysed either using Kruskal-Wallis *k*-sample test or Mann-Whitney *U*-test in conjunction with Tukey's multiple means comparison test.

All *Tisbe* data are presented in terms of equivalent density calculated as individuals per litre.

A3.2 Results

The origin of the salt source used in the culture media did not exert a significant effect on *Tisbe* productivity after nine days. Culture populations maintained in artificial seawater tended to exhibit slightly higher population densities than those maintained in natural seawater media (Table A.2.1). The highest maximum densities recorded for both artificial and natural seawater were from cultures maintained at 30 ‰. No significant differences were found between the productivities of *Tisbe* cultured at the five different salinities (p>0.05) producing a mean population density of 1,215 individuals L^{-1} ranging from 300 to 2,100 *Tisbe* L^{-1}.

There was no significant influence on population composition with ovigerous females comprising less than 1% of the population, male and immature copepodids 90% and nauplii 10% of the total numbers.

Table A3.1: Population densities (mean ±standard error) and composition for *Tisbe* cultures maintained in artificial and natural seawater of 25, 30, 35, 40 and 45 ‰.

	Natural Seawater		Artificial Seawater	
Salinity	Mean ±SE *Tisbe* L^{-1}	Range	Mean ±SE *Tisbe* L^{-1}	Range
25 ‰	1150 ±170	655 - 1435	1510 ±40	1420 - 1595
30 ‰	1115 ±280	300 - 1520	1530 ±305	835 - 2095
35 ‰	1020 ±175	540 - 1360	1360 ±155	775 - 1335
40 ‰	1155 ±130	850 – 1515	1400 ±150	1015 - 1680
45 ‰	1095 ±50	965 - 1215	1130 ±125	860 - 1460

The salinities of the cultures were consistent at the treatment level throughout the trial with all cultures experiencing an average temperature of 21.7 °C ±0.07 °C associated with a range of 20.6 to 22.4°C.

Salt source exerted a significant effect on the pH of the culture media with artificial sweater exhibiting a mean pH of 7.9±0.03 (7.6 to 8.2) compared with a mean pH of 8.1±0.01 (8.0 to 8.2) for natural seawater. The pH across the salinity range of 25 ‰ to 45 ‰ did not differ significantly within each salt source.

Mean dissolved oxygen levels recorded were not influenced by salinity level, salt source or interaction between the salinity and salt source. *Tisbe* cultures experienced mean percent dissolved oxygen levels of 86.4% ±0.84, associated with a range from 76.8 to 98.8%.

A3.3 Discussion

The use of artificial seawater offers benefits from the point of view of convenience and consistency of composition (Heinle, 1969), and the lack of contamination from metabolites present in natural seawater as a result of the presence of zooplankton species.

The marginal benefit arising from the use of artificial seawater in the small cultures is not sufficiently great to justify its use in culture systems requiring large volumes of saltwater. However where small differences in productivity are considered to be potentially important, artificial sea salts offer a means by which to minimise confounding influences.

A4 Assessment of additional diets

The influence of nine diet treatments were assessed in terms of the *Tisbe* population density supported after 6 days of culture.

A4.1 Materials and methods

The nine diets described in Table A4.1 were prepared using 0.2 µm filtered seawater at 35 ‰. The microalgae were obtained from axenic cultures maintained at 25 °C and a 14L:10D photoperiod (Section 2.2.2), and the artificial feeds of the shelf.

Three of the nine treatment diets are controls - *Tetraselmis, Isochrysis* and unfed - against which the performance of a previously untried alga *Nitzschia* and two artificial diets (Gibson salmon pellet and Lansy® fish crumble) were compared.

Table A4.1: A description of the components of the nine treatment diets assessed in terms of the total number of *Tisbe* developing in cultures.

Diet Label	Composition
Unfed	0.2 µm filtered seawater
Tet	*Tetraselmis* at a final density of $6x10^4$ cells mL^{-1}
Iso	*Isochrysis* at a final density of $1x10^5$ cells mL^{-1}
Nitz1	*Nitzschia* at a final density of $6x10^4$ cells mL^{-1}
Nitz2	*Nitzschia* at a final density of $1.2x10^5$ cells mL^{-1}
NRG1	50 grains of the artificial diet NRG4, weighing approximately 12 µg,
NRG2	100 grains of the artificial diet NRG4, weighing approximately 24 µg
Salm1	five ¼ pieces of size 2 mm salmon pellets
Salm2	five ½ pieces of size 2 mm salmon pellets

Thirty-six 250 mL cylindrical, clear plastic culture units were washed in hot water, rinsed and allowed to air-dry.

Four replicate culture units for each diet were inoculated with ten ovigerous *Tisbe* and randomly arranged on a shelf in a constant temperature room at 23 °C experiencing a 12L:12D photoperiod.

The cultures were left essentially untouched for the duration of the trial with all culture units monitored on days 3 and 6 for salinity, dissolved oxygen, pH and temperature levels using the equipment and techniques described previously in Section 2.2.2. General culture health was also appraised on the basis of *Tisbe* activity and the presence of ovigerous females.

On day 6 of the trial 5 mL of formalin was added to each culture unit. The immobilised copepods were then passed over a 44 µm screen to reduce sample volume and preserved in 40 mL of a 1:1:8 mixture of full strength formalin, glycerol and seawater.

Samples and subsequent data were treated in the same manner as described above in A3.1.

A4.2 Results

Diet exerted a significant ($p<0.01$) influence on *Tisbe* culture productivity as determined by the number of individuals developing over nine days. Algae fed cultures yielded an average 720 *Tisbe* L^{-1} ranging from 147 to 1,280 individual L^{-1}, compared with those cultures fed artificial diets which produced an average 215 *Tisbe* L^{-1} over the range from 0 to 765 individual L^{-1} (Figure A4.1).

The influence of diet on *Tisbe* culture population composition resembled that displayed by total numbers (Figure 4.4.2). Population composition data was unable to be transformed to meet the assumptions of ANOVA, with Kruskal-Wallis *k*-sample test unable to identify significant differences between the numbers of ovigerous females in the cultures. On average 1.5% of the total culture populations were ovigerous females, the overall range encompassing 0 to 25% of the populations. Table 4.2.1 summarises the effects of diet on population composition.

Table A4.2: The number of *Tisbe* nauplii (NI-VI), copepodids (CI-CVI) and ovigerous females (mean ±standard error) present after nine days in cultures fed one of nine diets and maintained at 22 °C and 35 ‰, and the proportion of the total population comprising ovigerous *Tisbe*.

Diet	Nauplii	Copepodids	Ovigerous	% ovigerous
Unfed	35 ±11	164 ±40	1.8 ±1.7	1.6 ±1.6
Salm 1	19 ±11	204 ±20	0	0
Salm 2	0	7 ±3	0	0
NRG 1	50 ±12	509 ±135	1.8 ±1.7	0.3 ±0.3
NRG 2	7 ±7	92 ±39	1.8 ±1.7	6.3 ±6.3
Nitz 1	73 ±13	600 ±70	13.5 ±3.8	2.2 ±0.8
Nitz 2	58 ±22	652 ±174	6.8 ±2.7	0.8 ±0.3
Tet	82 ±13	584 ±210	5.0 ±3.1	1.8 ±1.1
Iso	203 ±98	610 ±84	3.5 ±2.0	0.4 ±0.3

Culture temperature did not differ significantly ($p>0.05$) between culture units exhibiting a mean of 22.2 ±0.08 °C with an overall range of 21.4 to 24.3°C.

Dissolved oxygen and pH levels were found to differ significantly ($p< 0.01$) between diet treatments (Figure A4.3). The unfed control and all the algae fed *Tisbe* cultures exhibited higher culture pH levels. *Tisbe* cultures fed artificial diets recorded lower pH levels due to a lack of photosynthetic activity and concurrent breakdown of detritus. All cultures were within the range of 7.4 to 8.6 pH units.

Dissolved oxygen levels exhibited an overall range from 0.5 to 9.1 mgO_2 L^{-1}, corresponding to a range of 2 to 122 percent saturation, with a significant split between the unfed and algae fed cultures, and those fed artificial diets.

A4.3 Discussion

The benthic diatom *Nitzschia* was found to support *Tisbe* productivity comparable with that achieved using *Tetraselmis* and *Isochrysis*.

The population density equivalent to 1,280 *Tisbe* L^{-1} achieved on a diet of *Isochrysis* over nine days at 22 °C and 35 ‰ is similar to the results achieved in the temperature by salinity trial (Section 2.3.2.4). Cultures maintained at 20 °C produced around 900 individuals L^{-1} after nine days when fed a diet of mixed diet of *Isochrysis*, *Tetraselmis* and fish crumble.

The use of small volume static cultures exacerbated the water polluting effects of the artificial NRG4 and salmon feed diets. Using a smaller ration and implementing a degree of water exchange may have improved the productivity of the cultures.

The second trial assessing *Tisbe* performance on a variety of diets (Section 2.3.2.5) was designed to address these issues and used a reduced ration of artificial food in combination with a flow-through system. The revised system benefited from a slightly larger culture volume and the daily introduction of fresh food and clean culture medium (Fava & Crotti, 1979; Vijverberg, 1989).

The 500 mL culture units with a reservoir and screened overflow outlet may well have resulted in higher population densities than that achieved in the static cultures as a result of the dilution of growth inhibiting compounds and maintenance of food levels.

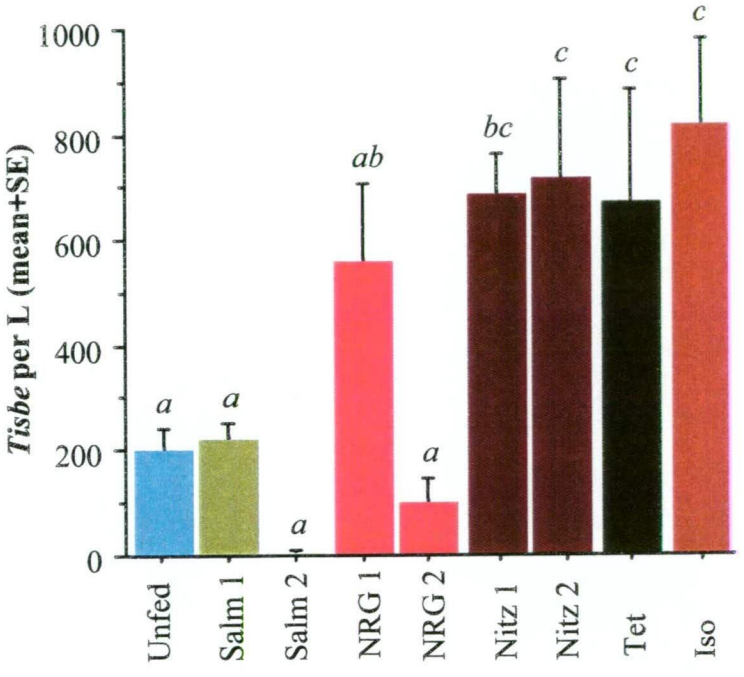


Figure A4.1: Influence of diet on the productivity of *Tisbe* as determined by the total number of individuals developing over nine days when maintained at 22°C in media of 35 ‰. Nine diets were assessed: 0.1 µm filtered seawater (unfed), salmon pellets at two levels (Salm 1 and 2), fish crumble at two levels (NRG 1 and 2), *Nitzschia* at two cell densities (Nitz 1 and 2), *Tetraselmis* (Tet) and *Isochrysis* (Iso). Italicised superscripts indicate significanlty different means ($p<0.01$) as identified by ANOVA of square root transformed data and Scheffe's multiple means comparison test.

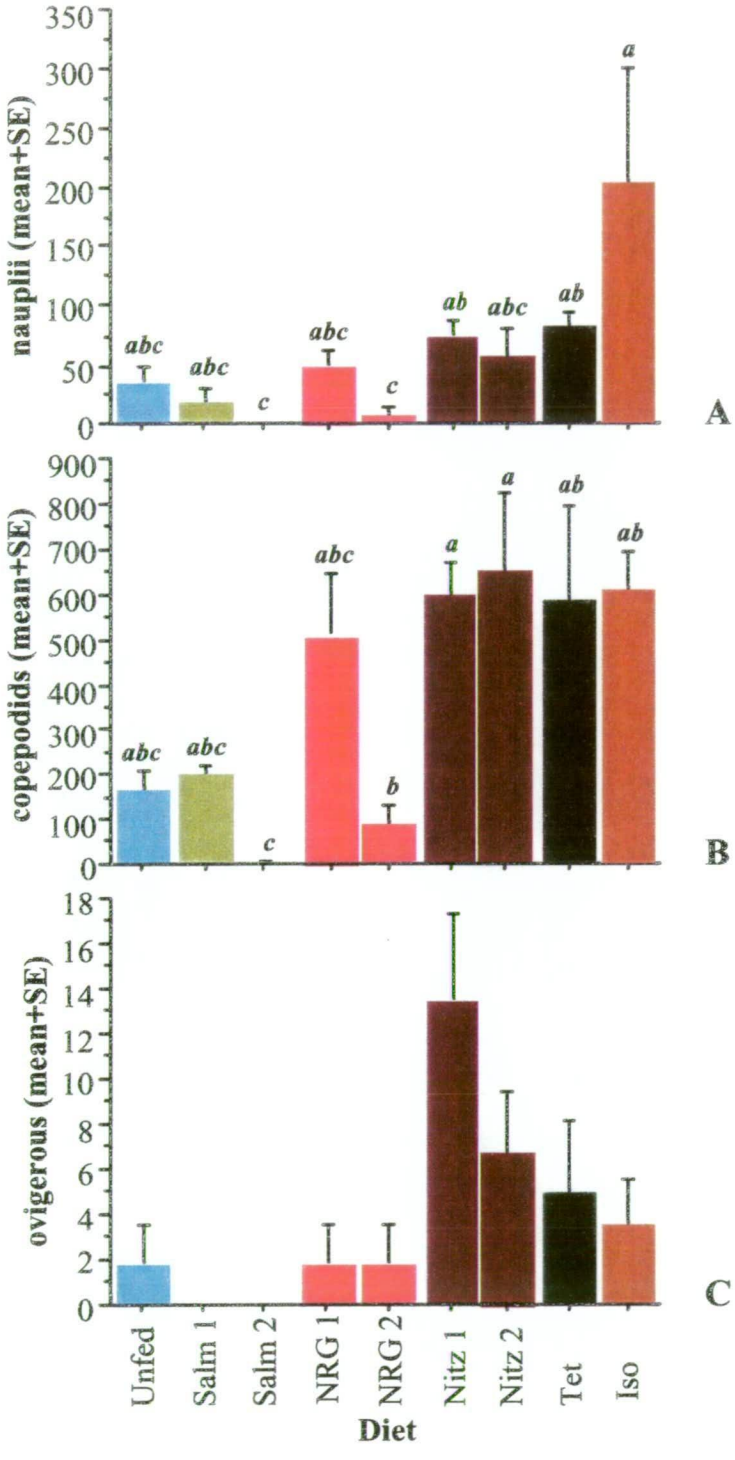


Figure A4.2: Influence of diet on the population composition of *Tisbe* cultures at 22 °C in media of 35 ‰ fed one of nine diets: 0.1 µm filtered seawater (unfed), salmon pellets at two levels (Salm 1 and 2), fish crumble at two levels (NRG 1 and 2), *Nitzschia* at two cell densities (Nitz 1 and 2), *Tetraselmis* (Tet) and *Isochrysis* (Iso). Italicised superscripts indicate significantly different means ($p<0.05$) as identified by Kruskal Wallis *k*-sample and Tukey's multiple means comparison tests. No significant differences were identified in the number of eggs present between cultures (C).

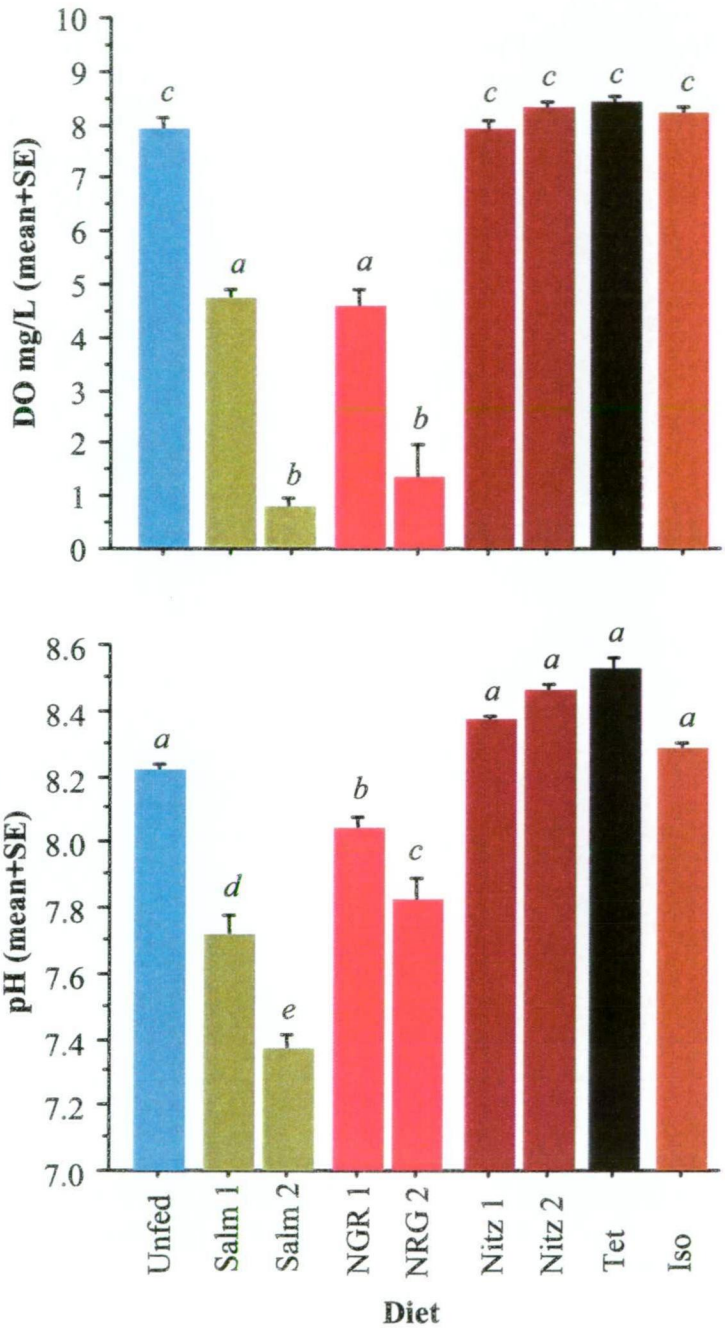


Figure A4.3: *Tisbe* culture medium dissolved oxygen and pH levels after six days maintained at 22 °C and 35 ‰ on nine diets: 0.1 µm filtered seawater (unfed), salmon pellets at two levels (Salm 1 and 2), fish crumble at two levels (NRG 1 and 2), *Nitzschia* at two cell densities (Nitz 1 and 2), *Tetraselmis* (Tet) and *Isochrysis* (Iso). Italicised superscripts indicate significantly different means ($p<0.05$) as identified by analyses of variance and Scheffe's multiple means comparison tests.

A5. Summary of environmental parameters recorded for experiments detailed in Chapter 2

A5.1 The effects of salinity and temperature (Section 2.3.2.4)

Table A5.1.1: Water bath temperatures (°C mean, standard errors (SE) and ranges) experienced by experimental *Tisbe* cultures over the nine day duration of the temperature - salinity interaction trial.

Treatment (°C)	Mean (°C)	SE (°C)	Minimum (°C)	Maximum (°C)
10	10.2	0.07	9.8	10.8
15	14.9	0.02	14.8	15.1
20	20.0	0.03	19.8	20.2
25	25.1	0.04	24.9	25.5
30	30.5	0.05	30.0	30.7

Table A5.1.2: Salinities (‰, mean, standard errors (SE) and ranges) recorded for experimental *Tisbe* cultures at the termination of the temperature - salinity interaction trial.

Salinity (‰)	Mean (‰)	SE (‰)	Minimum (‰)	Maximum (‰)
15	15.2	0.09	15	16
20	20.7	0.18	20	22
25	23.8	0.28	21	25
30	28.9	0.63	27	30
35	34.8	0.19	34	37
40	39.2	0.21	38	40

Table A5.1.3: Dissolved oxygen values (mean, standard errors (SE) and ranges) recorded for experimental *Tisbe* cultures at the termination of the temperature - salinity interaction trial.

Temperature (°C)	DO ±SE (mg O_2 L^{-1})	Range (mg O_2 L^{-1})	DO ±SE (%O_2)	Range (%O_2)
10	8.6±0.12	7.3 - 9.3	89±0.8	76-93
15	7.1±0.11	5.9 - 7.9	81±1.0	75-89
20	6.7±0.10	5.7 - 7.4	86±1.0	74-98
25	6.3±0.10	5.0 - 6.9	86±0.9	80-92
30	5.8±0.09	5.1 - 6.5	86±1.0	78-93

Table A5.1.4: Final pH values mean, standard errors (SE) and ranges) recorded for experimental *Tisbe* cultures at the termination of the temperature - salinity interaction trial.

Treatment (°C)	Mean pH	SE	Minimum pH	Maximum pH
10	8.07	0.11	7.34	9.24
15	8.69	0.12	7.15	9.33
20	7.88	0.11	7.26	8.94
25	8.14	0.12	7.07	8.92
30	8.12	0.08	7.28	8.65

A5.2 The effect of diet (Section 2.3.2.5)

Table A5.2.1: Dissolved oxygen levels (mean mgO_2 L^{-1} ±standard error) recorded for *Tisbe* cultures fed different diets at 20°C and 35 ‰ for 14 days. Different scripts indicate significant differences. Mix – mixture of *Isochrysis*, *Nitzschia* and *Tetraselmis*; T+I - *Tetraselmis* and *Isochrysis*; Nz - *Nitzschia*; All - the three microalgae plus fish crumble; NRG - Lansy® fish crumble; Unfed - filtered seawater in Tables A5.5 to A5.7.

Diet	Mean ±SE	Lowest	Highest	$p<0.05$
Mix	6.2 ±0.14	3.4	10.6	*abd*
T+I	7.1 ±0.16	3.3	11.2	*d*
Nz	4.9 ±0.18	1.2	10.4	*c*
All	6.5 ±0.14	2.6	10.6	*bd*
NRG	5.9 ±0.10	2.7	9.4	*b*
Unfed	6.9 ±0.08	4.9	9.7	*ab*

Table A5.2.2: Final culture media salinity levels (mean ‰ ±standard error, range) recorded for *Tisbe* cultures fed different diets at 20°C for 14 days. Different scripts indicate significant differences.

Diet	Mean ±SE	Lowest	Highest	$p<0.01$
Mix	37.8 ±0.11	37.5	38.0	*ab*
T+I	37.7 ±0.37	36.5	39.0	*ab*
Nz	37.5 ±0.22	36.5	38.0	*ab*
All	38.0 ±0.13	37.5	38.5	*b*
NRG	36.8 ±0.34	35.5	38.0	*a*
Unfed	37.3 ±0.11	37.0	37.5	*ab*

Table A5.2.3: Final culture media pH levels (mean ±standard error, range) recorded for *Tisbe* cultures fed different diets at 20°C and 35 ‰ for 14 days. Different scripts indicate significant differences.

Diet	Mean ±SE	Lowest	Highest	$p<0.01$
Mix	7.57 ±0.050	7.45	7.75	*ab*
T+I	7.68 ±0.037	7.58	7.82	*bc*
Nz	7.64 ±0.015	7.58	7.68	*b*
All	7.48 ±0.022	7.41	7.55	*a*
NRG	7.79 ±0.020	7.72	7.85	*cd*
Unfed	7.90 ±0.013	7.87	7.94	*d*

A6. Summary of larval flounder length measurements recorded for feeding trials in Section 2.3.3.5.

Table A6.1.1: Average number of feed items (mean larva^{-1} ±SE, n=75) in the gut contents of flounder at five age classes.

Age (dph)	rotifers	Artemia	Tisbe
5	0.5 ±0.11	0	5.2 ±0.47
12	9.8 ±0.52	0	3.2 ±0.43
19	16.9 ±1.03	4.4 ±0.59	2.4 ±0.33
26	20.8 ±1.63	8.3 ±1.00	1.0 ±0.24
33	32.9 ±3.07	18.2 ±1.09	7.5 ±5.2

Table A6.1.2: Mean standard larval lengths with standard error (SE) for the five age groups of flounder investigated. All means were significantly different ($p<0.05$) between age groups as identified by ANOVA and Scheffe's multiple means comparison.

Age (dph)	Mean (mm)	SE	n
5	2.92	0.006	375
12	3.37	0.011	350
19	4.55	0.022	373
26	5.56	0.027	374
33	6.74	0.038	344

Table A6.1.3: Average larval widths with standard error (SE) for the five age groups of flounder investigated. All means were significantly different ($p<0.05$) between age groups as identified by ANOVA and Scheffe's multiple means comparison.

Age (dph)	Mean (µm)	SE	n
5	165	0.5	375
12	275	2.4	350
19	739	8.6	374
26	902	11.5	374
33	1948	17.3	343

Table A6.1.4: Average larval jaw lengths with standard error (SE) for the five age groups of flounder investigated. All means were significantly different ($p<0.05$) between age groups as identified by ANOVA and Scheffe's multiple means comparison.

Age (dph)	Mean (µm)	SE	n
5	325	1.1	375
12	408	1.6	347
19	569	2.9	374
26	821	4.2	374
33	791	4.9	344

Appendix B
Apocyclops

B1 Introduction

Commercial growers of barramundi in the Northern Territory of Australia employ extensive green-water culture techniques in ponds for rearing larvae and juveniles (Schipp, 1996). This method involves the fertilisation of natural waters to encourage the development of a bloom of native algae and subsequent zooplankton species. Water entering the earthen ponds is filtered down to 250 µm to exclude large zooplankton and fauna, the subsequent plankton blooms exhibiting large variation in species composition (Jeff Reid, Barramundi Farms NT, *pers. comm.*).

The use of large ponds in the extensive rearing of fish larvae has met with varying degrees of success, but where successful the resultant larvae have proven far superior to hatchery-reared larvae in terms of the growth achieved and stress tolerance (Jeff Reid, Barramundi Farmers NT, *pers. comm.*). The differences have been attributed to the more nutritious natural diet comprised largely of copepod species in addition to other zooplankton constituents such as rotifers and polychaete larvae available to the developing larvae.

Despite increased consistency between production run, there is still an element of unreliablity in the prediction of the degree of success or failure of any particular cohort of fish larvae. It is assumed that the difficulties are largely due to:

- inconsistency of zooplankton populations to provide suitable food for the fish larvae
- predation upon the fish larvae by components of the zooplankton.

This investigation was conducted to gain some insight into the natural cycle of zooplankton populations of farm ponds with the view of optimising the time at which fish larvae may be introduced to ponds to maximise food suitability and availability, and minimise the potential for copepod predation on larvae.

The changes observed in the zooplankton communities of six farm ponds monitored over a period of four weeks are presented, accompanied by a record of changes in environmental parameters. The main zooplankton components were enumerated with more attention directed at the copepod element due to the current interest in these crustaceans as a fundamental component of larval finfish diets. An attempt is made to elucidate relationships between dominant zooplankton species and prevailing environmental conditions.

The information in this chapter is a consolidated version of the information distributed to barramundi farmers in the Northern Territory and provides background information on the general conditions likely to be encountered in extensive larval rearing ponds established in the Darwin area. The study also provided the impetus to further investigate the potential of *Apocyclops dengizicus* as a live food for larviculture.

B2 Materials and methods

The six ponds monitored were located a half hour drive 50 km south-west of Darwin on Barramundi Farms NT. Every Monday and Thursday (dates and corresponding sample numbers listed in Table B2.1) between 08:00 and 11:00 hours during the month of November 1995 zooplankton samples were collected and temperature, salinity, dissolved oxygen, turbidity, light, pH and nitrogen levels recorded for each pond. All six ponds were sampled from under their respective automatic feeders suspended above the water on a platform, which were not in use, approximately 1m from the side of the pond. The sites and depths for sampling were chosen for consistency and ease of sampling.

Table B2.1: Sampling dates during November 1995 and the corresponding sample numbers used in following figures and tables.

Sample Number	Date	Sample Number	Date
1	7 November	5	19 November
2	9 November	6	23 November
3	13 November	7	27 November
4	16 November	8	30 November

Surface water temperature and dissolved oxygen (DO) were measured *in situ* at a depth of 10 cm using a portable WTW Oxi 320 meter. Salinity was assessed using an ATAGO S-10 hand held refractometer with turbidity recorded as secchi disk depth. Light levels were measured at each of the six ponds at the water surface during the 3 hour sampling period using a portable DSEQ 1400 lux meter.

A one litre water sample was taken from 10 cm below the surface of each pond and immediately put on ice for pH, ammonia and nitrite determination at DAC using Hanna instruments pH meter HI 8424, and the appropriate HACH colourimetric test kits.

Zooplankton samples were collected using a 76 µm mesh plankton net dragged just below the water surface for five 1m sweeps, corresponding to a sample volume of 350 L. Concentrated samples were transferred to 70 mL specimen bottles and put on ice until formalin and glycerol could be added to achieve a final 1:1:3 mixture of formalin, glycerol and water for long term preservation.

The faunal composition of each zooplankton trawl was assessed by separating and counting the various components identified in four 1 mL samples taken from well mixed contents of the 70 mL specimen bottles. The three dominant zooplankton classes assessed included copepods (Copepoda), polychaete larvae (Polychaeta) and rotifers (Rotifera). The copepod fraction of the zooplankton population was further sorted into adults (CVI) belonging to the three orders Calanoida, Cyclopoida and Harpacticoida, the intermediate copepodid stages (CI-CV) and naupliar stages (NI-NVI). Enumeration was completed using a stereo SZH Olympus dissecting microscope with sub-stage illumination and a magnification range of 7.5x to 64x.

Representative samples of the five major copepod groups recognised were sent to Dr David McKinnon (Australian Institute of Marine Science, Townsville, Queensland) for identification.

Background history of the ponds:

Pond 5:

A 0.2 ha pond in which maturing prawns had been held for 20 weeks with constant water flow which was drained and harvested prior to the final sampling date.

Pond 14:

A 0.2 ha pond stocked with 6 000 barramundi in March 1995, fed by automatic feeders with constant water flow. Harvested between samples six and seven.

Pond 27:

A 0.4 ha pond stocked with 40 000 barramundi in February 1995, fed by automatic feeders with constant water flow.

Ponds 10, 19 & 26:

Three 0.1 ha ponds undergoing the process of being 'bloomed-up', initiated during the first week of November 1995: each pond was filled with a mixture of freshwater and native screened seawater containing natural zooplankton entering at approximately 35‰ to which was added 16 kg of diammonium phosphate (DAP) and 3 kg of urea. A further 8 kg of both DAP and Urea were added to each pond one week later. The ponds were stocked with prawn post larvae stage 15 between samples 4 and 5 on November 17. The ponds were operated as static systems with no water flow through. Aeration was supplied by paddlewheels when required.

B3 Results

B3.1 Zooplankton populations

Pond 19 was the most productive supporting 3 720 zooplankters L^{-1}, approximately double the density of that supported by ponds 5, 10 and 26, and 10 orders of magnitude more greater than ponds 14 and 27 (Figure B3.1).

Pond 19 supported the greatest number of copepods at 904 L^{-1} comprising 24.3% of the zooplankton, the remainder being rotifers. Ponds 5, 10 and 26 supported around 2 000 zooplankters L^{-1} although they differed in the proportions of copepods and rotifers supporting 2.2% (40 L^{-1}), 22.8% (460 L^{-1}) and 43.6% (660 L^{-1}) copepods respectively, the remainder being rotifers.

Ponds 14 and 27 differed from the other four ponds in both their zooplankton density and faunal compositions. Pond 14 supported 220 zooplankters L^{-1}, only 21.8% of these were copepods (50 L^{-1}), the remainder being polychaete larvae (170 L^{-1}).

Pond 27, the least productive carrying 110 zooplankters L^{-1}, is notable as the only pond to support representatives from all three faunal groups. The zooplankton population comprised 63.8% copepods (70 L^{-1}), 27% rotifers (30 L^{-1}) and 9.6% polychaete larvae (10 L^{-1}).

By focusing on the copepod portion of the zooplankton populations further differences between ponds become evident. Figure B3.2 presents the mean percentage compositions of the pond copepod populations. Ponds 5, 14 and 27 were dominated by nauplii over the four weeks. Ponds 10 and 19 supported a copepod population comprising approximate equal proportions of nauplii, copepodids (CI-CV) and reproductive adults (CVI); with the copepod component of the zooplankton of pond 26 largely dominated by the intermediate copepodid life stages.

The proportional contribution of copepod class representatives amongst the adult copepods revealed further distinctions and similarities between ponds (Table B3.1), the most obvious being the dominance of the copepod populations by calanoids in five out of the six ponds, and the presence of harpacticoids in only two of the ponds. Pond 5 was the only pond not dominated by calanoid copepods, harpacticoids being the most common copepod. Cyclopoids were present in all ponds.

The zooplankton populations of ponds 14 and 27 appeared to fluctuate moderately around a low level, whereas the zooplankton populations of ponds 5, 10, 19 and 26 exhibited

larger fluctuations in numbers. The difference may be attributed to the different pond maintenance regimes implemented and the more open nature of ponds 14 and 27 which would more closely reflect natural zooplankton densities.

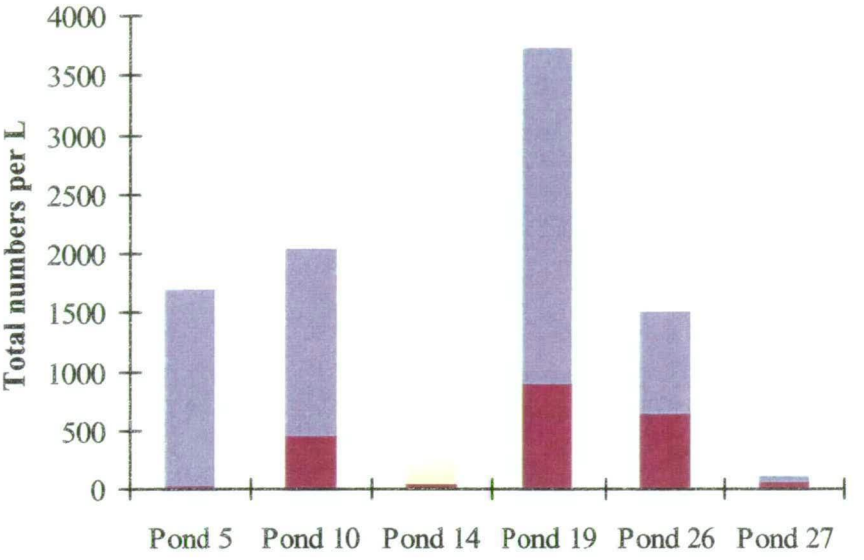


Figure B3.1: Comparison of pond zooplankton population densities in terms of ■ copepod, ■ polychaete and rotifer ■ representatives.

Table B3.1: Pond zooplankton adult copepod (CVI) percentage distribution between three Orders represented within the Class Copepoda.

Pond	Calanoida	Harpacticoida	Cyclopoida
5	19	51	30
10	92	0	8
14	85	0	15
19	98	0	2
26	90	5	5
27	98	0	2

The total number of copepod life stages enumerated from each pond at each sampling revealed ponds 10, 19 and 26 exhibited a strong peak in copepod numbers, greater than 250 copepods L^{-1} (Figure B3.3). Conversely ponds 5, 14 and 27 supported populations of less than 50 copepods L^{-1} which did not express such obvious increases in numbers.

The two ponds supporting polychaete larvae exhibited progressive increases in numbers of polychaete larvae with either a weekly cycle (pond 14) or a two weekly cycle (pond 27). These two ponds were exposed to flow through conditions with average salinities of 31 and 30‰ respectively.

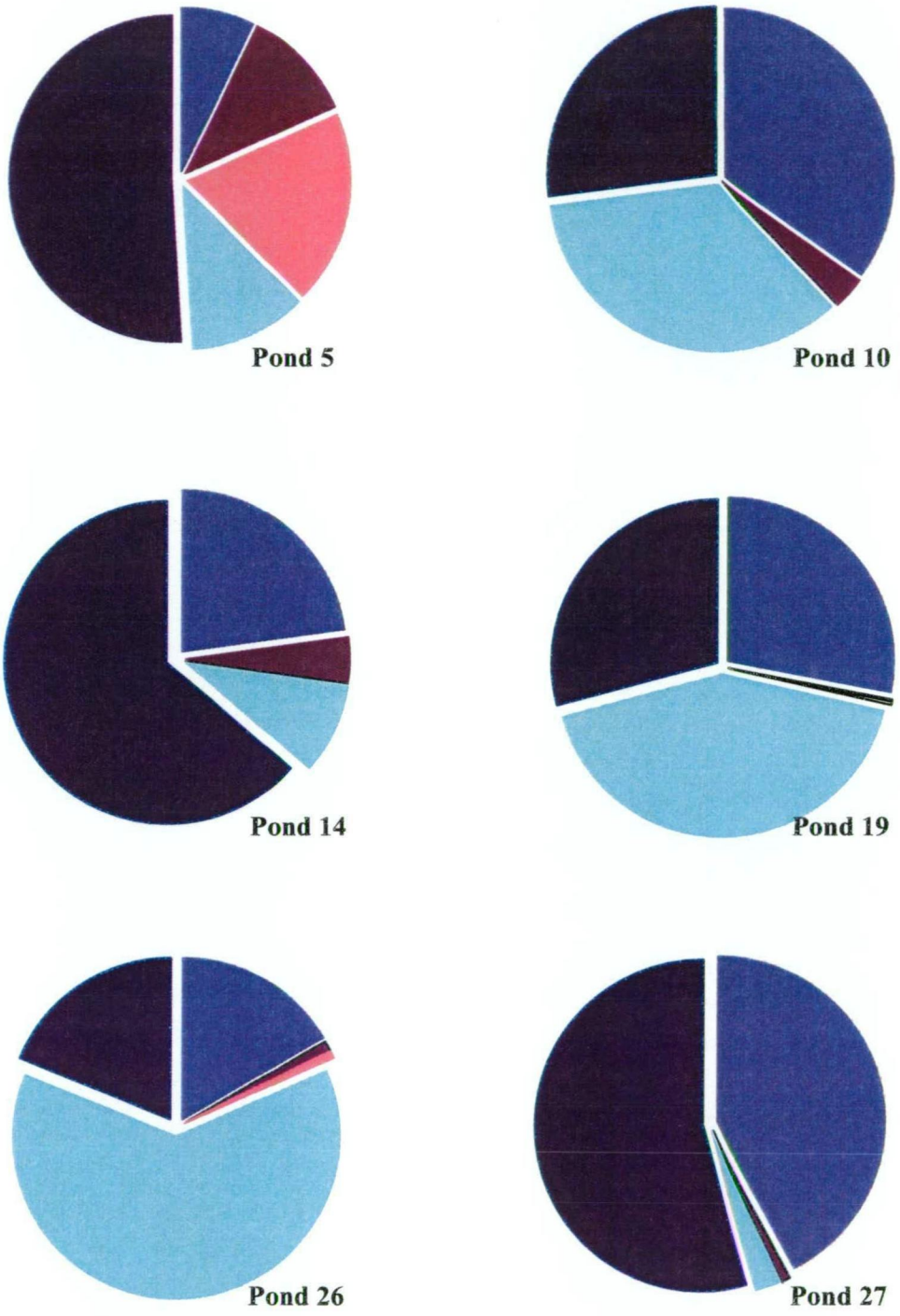


Figure B3.2: Relative composition of the copepod component of green -water pond zooplankton populations sampled from Reid's Barramundi Farm during November 1995. ■ nauplii (all NI through NVI), ■ copepodids (all CI through CV), ■ calanoids, ■ cyclopoids and ■ harpacticioids.

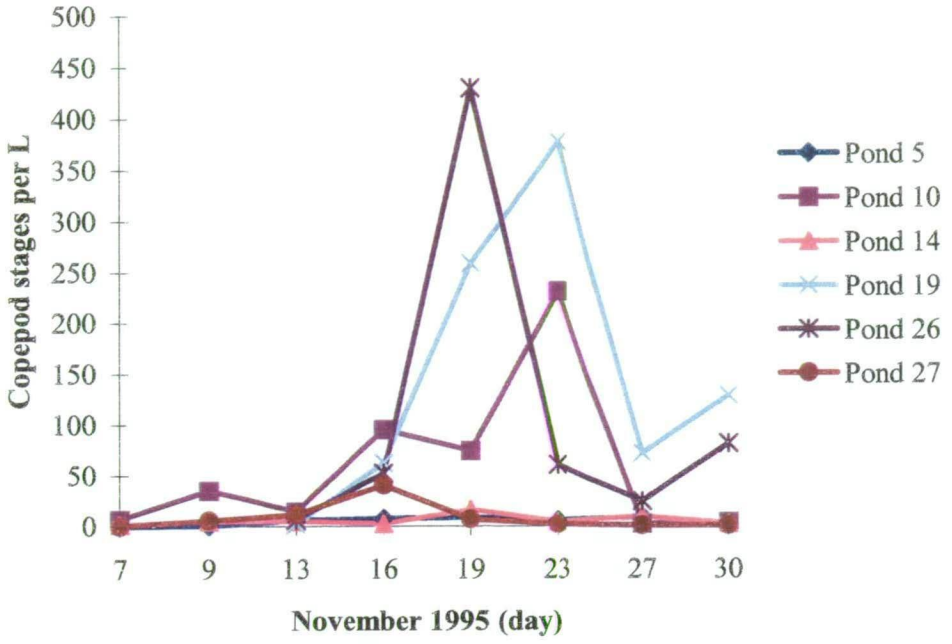


Figure B3.3: Temporal variation in total copepod numbers between sampling dates in each of the six ponds at Barramundi Farms NT.

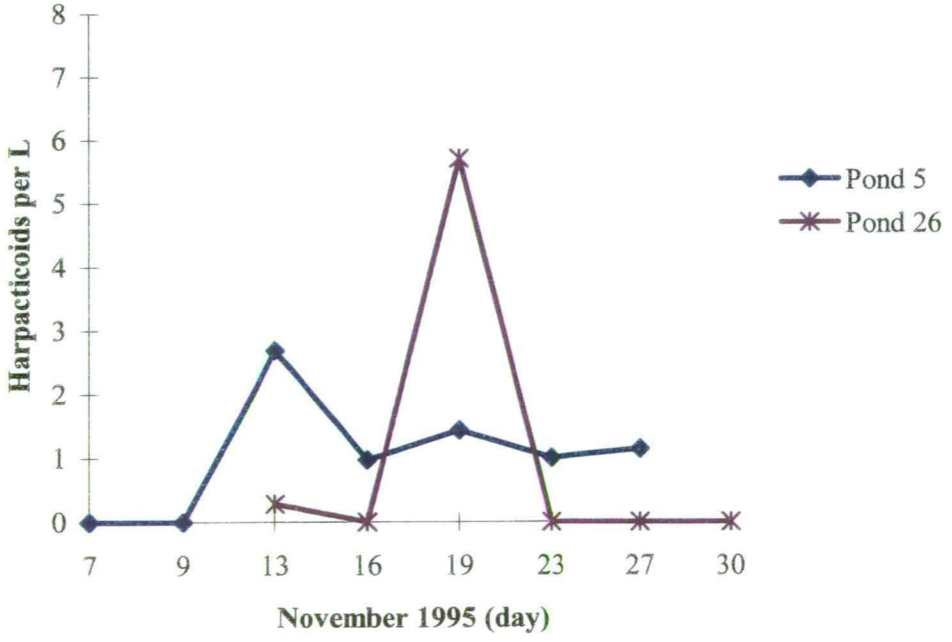


Figure B3.4: Temporal variation in total harpacticoid numbers between sampling dates in each of the six ponds at Barramundi Farms NT.

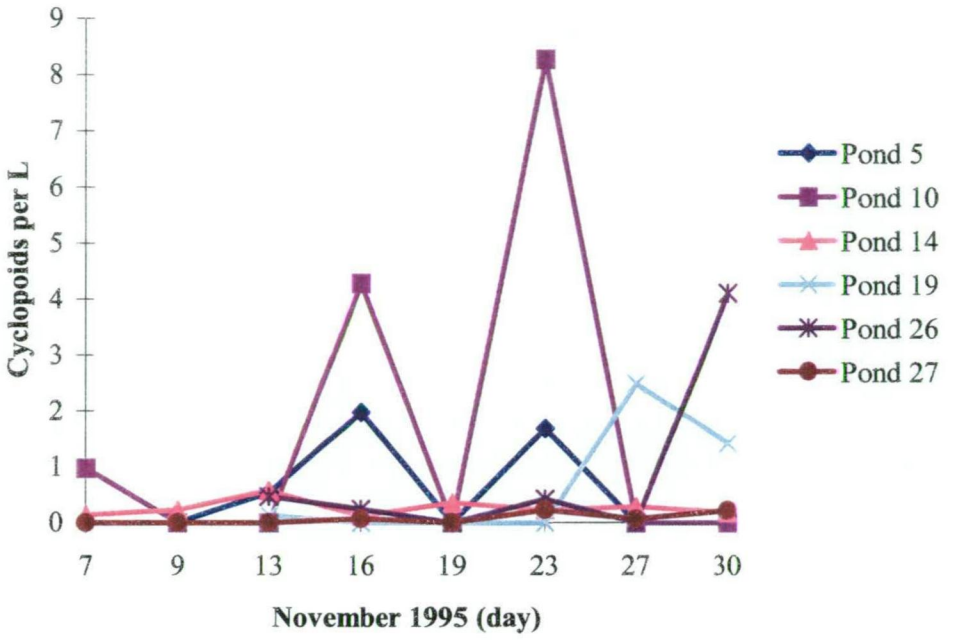


Figure B3.5: Temporal variation in total cyclopoid numbers between sampling dates in each of the six ponds at Barramundi Farms NT.

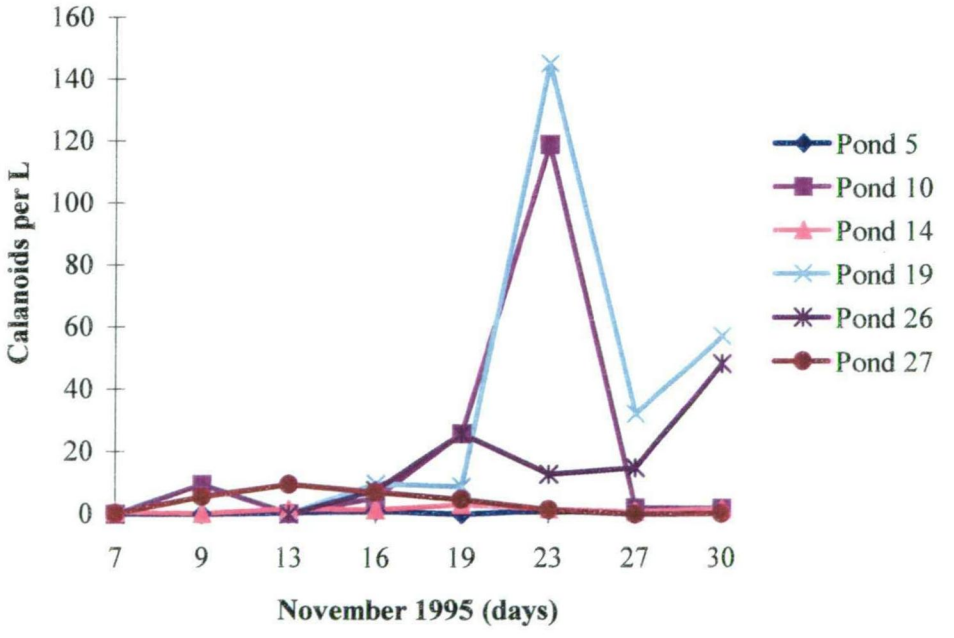


Figure B3.6: Temporal variation in total calanoid numbers between sampling dates in each of the six ponds at Barramundi Farms NT.

Pond 14 is the only pond not to have a rotifer zooplankton component. The rotifers of pond 27 were present at low levels, usually less than 1 L^{-1}, only showing an increase to 26 L^{-1} after sample 7 which coincided with a decline in copepod numbers. Ponds 10 and 26 exhibited a slow general increase in rotifer numbers to densities of 600 and 250 rotifers L^{-1} respectively. Pond 19 on the other hand exhibited a constant increase in rotifer densities to 1200 L^{-1} at the final sampling. Pond 5, behaved in a manner similar to pond 19, with rotifer numbers generally increasing to peak at a density of 1000 rotifers L^{-1} prior to draining.

Cyclopoid and harpacticoid copepod species were poorly represented, adults occurring at densities of less than 10 L^{-1} when present, in comparison to calanoid numbers, which were generally higher, ranging from 20 to 160 L^{-1} in ponds 10, 19 and 26. Harpacticoid species were only seen in samples collected from two of the six ponds (Figure B3.4), cyclopoid species being more common exhibiting an approximate fortnightly cycle in all ponds (Figure B3.5).

Calanoid densities peaked in ponds 10, 19 and 26 three weeks into the four week sampling run, and showed signs of another peak within two weeks (Figure B3.6). These three ponds were in the process of being 'bloomed-up' as compared to ponds 5, 14 and 27 which maintained populations of calanoids below 10 L^{-1}, and did not show large peaks in numbers.

B3.2 Environmental parameters

All six ponds exhibited similar surface water temperature fluctuations within the range of 28 to 33.7°C (Table B3.2) where overcast mornings were associated with lower temperature readings.

Pond salinities were found to differ quite markedly with large fluctuations experienced in ponds 5, 10, 19 and 26 compared to ponds 14 and 27 (Table B3.3). Ponds 14 and 27 exhibited a consistently higher salinity than ponds 5, 10, 19 and 26, with the widest salinity ranges experienced in ponds 5 and 10. Pond 19 exhibited relatively consistent salinities in the lower mid-range around 12‰.

The pattern observed in pond salinities was mirrored in pH levels, pH being consistently lower in ponds 14 and 27 (below pH 8.0) with ponds 10, 14 and 26 exhibiting more variable profiles above pH 8.0 (Table B3.4). Pond 5 exhibited a unique pH profile over the four weeks commencing with a pH similar to ponds 14 and 27, increasing to levels comparable to the other three ponds within 3 weeks.

Dissolved oxygen levels (DO) also differentiated ponds 14 and 27, from ponds 5, 10, 19 and 26 (Table B3.5). The lower DO levels generally exhibited by ponds 14 and 27 corresponded to comparatively more turbid water (Figure B3.7). Light levels recorded during the eight 3 hour sampling periods ranged from 2 100 to 10 900 lux across all six ponds reflecting the variability in cloud cover during the beginning of the tropical wet season and natural light increase in light intensity between 08:00 and 11:00 hours.

In all six ponds total nitrogen levels were found to be undetectable or below recommended maximum levels (0.5 mg L^{-1}, Forteath, 1990). The highest total ammonia value measured, 0.4 mg L^{-1} in pond 26 during sampling six, was below the recognised 0.5 mg L^{-1} concern value (Table B3.6). Nitrite was similarly undetectable or present at low levels with the exception of pond 10. The levels of nitrite measured in pond 10 rose from 0.1 to 5.9 mg L^{-1} over the four week period (Table B3.7). Although obviously elevated in comparison to the other five ponds, this elevated level was of no great concern as it is well below the 20 mg L^{-1} considered to be threatening to fish health (Forteath, 1990) and 15 mg L^{-1} level, which can result in increased crustacean mortalities (Forteath, 1990).

Table B3.2: Average surface water temperatures (mean ±standard error) recorded in the six ponds monitored during November 1995.

Pond Number	Temperature (°C)	Minimum (°C)	Maximum (°C)
5	31.0 ±0.47	29.0	32.5
10	31.3 ±0.65	28.0	33.3
14	30.9 ±0.62	28.0	33.2
19	30.8 ±0.74	28.0	32.1
26	31.2 ±0.77	28.0	32.8
27	31.3 ±0.59	28.0	33.7

Table B3.3: Average surface water salinities (mean ±standard error) recorded in the six ponds monitored during November 1995.

Pond Number	Salinity (‰)	Minimum (‰)	Maximum (‰)
5	20.8 ±2.47	13.0	30.0
10	19.3 ±1.51	10.0	25.0
14	31.3 ±0.56	29.0	34.0
19	11.3 ±0.89	9.0	14.0
26	20.8 ±1.40	15.0	25.0
27	31.3 ±0.59	28.0	33.7

Table B3.4: Average surface water pH levels (mean ±standard error) recorded in the six ponds monitored during November 1995.

Pond Number	pH	Minimum pH	Maximum pH
5	8.2 ±0.21	7.6	8.8
10	9.0 ±0.18	8.6	9.9
14	7.7 ±0.12	7.3	8.3
19	8.8 ±0.14	8.4	9.3
26	9.1 ±0.17	8.7	9.8
27	7.9 ±0.07	7.7	8.1

Table B3.5: Average surface water dissolved oxygen levels (mean DO ±standard error) recorded in the six ponds monitored during November 1995.

Pond Number	DO ($mgO_2\ L^{-1}$)	Minimum DO	Maximum DO
5	8.0 ±0.65	5.8	10.5
10	7.6 ±0.33	6.2	8.9
14	6.6 ±0.62	4.2	9.2
19	7.1 ±0.35	5.5	7.9
26	7.9 ±0.39	7.0	9.3
27	31.3 ±0.59	28.0	33.7

Table B3.6: Average surface water levels of unionised ammonia (mean $mgNH_3\ L^{-1}$ ±standard error) recorded in the six ponds monitored during November 1995.

Pond Number	Ammonia ($mgNH_3\ L^{-1}$)	Minimum	Maximum
5	0.1 ±0.05	0.0	0.1
10	0.1 ±0.08	0.0	0.2
14	0.1 ±0.08	0.0	0.2
19	0.1 ±0.04	0.0	0.1
26	0.2 ±0.11	0.0	0.4
27	0.1 ±0.08	0.0	0.2

Table B3.7: Average surface water nitrite levels (mean $mgNO_2\ L^{-1}$ ±standard error) recorded in the six ponds monitored during November 1995.

Pond number	Nitrite ($mgNO_2\ L^{-1}$)	Minimum	Maximum
5	0.0 ±0.01	0.0	0.1
10	2.1 ±0.77	0.1	5.9
14	0.0 ±0.02	0.0	0.1
19	0.0 ±0.01	0.0	0.1
26	0.3 ±0.07	0.1	0.4
27	0.0 ±0.00	0.0	0.0

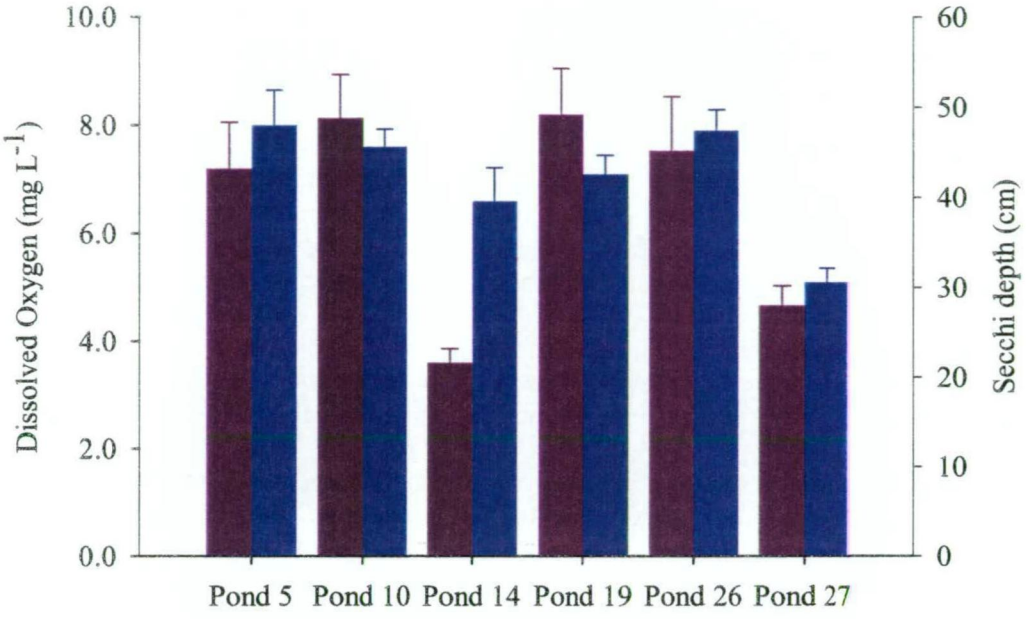


Figure B3.7: Average dissolved oxygen and pond turbidity levels (mean +standard error) recorded for the month of November 1995. Dissolved oxygen levels ■ were measured, and reported, in milligrams dissolved oxygen per litre (DO mg L^{-1}) using an WTW Oximeter. Turbidity levels ■ were measured in secchi depths recorded in centimeteres (cm).

B4 Discussion

Rotifers, primarily *Brachionus rotundiformus*, were the numerically dominant taxa in the ponds being 'bloomed-up', reflecting the immediate advantage of parthenogenic reproduction and short-life cycles (Geiger, 1983). Three days after rotifers peaked, copepod numbers first peaked. Copepods comprising five main species were the second most common taxa, while polychaete larvae featured in only one of the six ponds investigated.

This study was conducted primarily to obtain background information on the faunal composition of extensive tropical aquaculture pond zooplankton communities, the periodicity of copepod life stages, and to gain some insight to environmental conditions prevailing in tropical coastal aquaculture ponds in the Northern Territory. In the context of this thesis, copepods receive the most attention to the exclusion of rotifers and polychaete representatives.

Calanoid representatives were notable for their numeric dominance over a wide range of environmental conditions. The dominant calanoids were identified as two species of the genus *Pseudodiaptomus* which may account for the relatively abundant calanoid numbers over the range of environmental conditions observed.

The presence of the harpacticoid *Euterpina acutifrons* was associated with consistent mid-low range salinity (15-25‰), relatively consistent pH and high DO levels. Rotifers were the only other major zooplankton constituent enumerated under similar conditions.

No such relationship was obvious in the case of the cyclopoids, however a marine cyclopoid *Oithona* sp and a member of the Cyclopidae (later identified as *Apocyclops dengizicus*) were identified in the samples which may have confounded the recognition of any environmental preferences expressed by the different species of cyclopoids. It should be noted that cyclopoid numbers were elevated in ponds where calanoid numbers were lower.

Throughout the analyses two groups of ponds were consistently recognised: those ponds established longer than 6 months, and those in the process of being 'bloomed-up' for stocking with juvenile animals. The established ponds operated on a flow-through system supporting less dense zooplankton populations which did not exhibit strong peaks and declines in numbers in contrast to the ponds being 'bloomed-up'.

A two week cycle would appear to be common across all six ponds with nauplii appearing one to two weeks prior to adults, providing a one week window for stocking fish larvae to avoid the perceived predation or aggravation of the introduced larvae by adult copepods.

Brummett & Mattson (1996) completed a preliminary pond trial to monitor the periodicity of zooplankton size classes suited to larval fish production. They identified a 30 day cycle in ponds experiencing limited flow and regular addition of dry grass. The 30 day cycle was unrelated to the mean generation times of the taxa. Rotifers were observed to peak first followed by copepod species. It was recommended that thorough study of dynamics of zooplankton tropical ponds including analysis of population structure at the species level and more detailed study of phytoplankton and conservative water quality parameters was required to identify factors regulating zooplankton community composition and density.

Colura et al. (1987) investigated the effect of pond salinity on the zooplankton communities developing in unstocked fertilised mariculture ponds. Zooplankton densities were observed to peaked earlier in ponds at 10‰ due to rotifer blooms. *Acartia tonsa* was most dense in ponds at 20‰ averaging 128 L^{-1}, *Oithona* species at 15‰ averaging 34 L^{-1}, and polychaete larvae most abundant at 10‰ averaging 50 L^{-1}. *A. tonsa* numbers were highest in the absence of *Oithona*, and vice versa, similar to the observation in this study that cyclopoids were abundant only when calanoid species were low in the ponds monitored.

Relatives of the five most dominant copepod species observed have all been associated with aquaculture to some extent (Table B4.1). Despite the potential for the culture of *Pseudodiaptimus* as a result of the species tolerance of a wide range of environmental conditions, some concern existed with respect to the presence of prominent feruncular spine which may have caused difficulties in feeding by golden snapper larvae. In Thailand where the dominant copepod species in semi-intensive larval rearing ponds are *Acartia*, *Pseudodiaptomus* and *Oithona*, red snapper larvae targetted nauplii of *Acartia* and *Oithona* (Singhagraiwan & Doi 1993), possibly as a result of the extended feruncular setae.

The ponds studied at Barramundi Farms NT provided the rotifer stocks for cultures maintained at the Darwin Aquaculture Centre (DAC). The persistence of *Apocyclops dengizicus* in these cultures suggested potential ease of intensive culture of the copepod for use as a live food. Congers of *A. dengizicus* had been used in mariculture with some degree success, especially as a replacement for *Artemia* (James & Al-Khars, 1984), which encourgaed further investigation into the Darwin species of *Apocyclops*.

Garcia & Alejandre (1995) documented the pisciverous nature of the marine cyclopoids *Corycaeus truckicus* and *C. japnonicus* on the Pacific sardine. This report combined with the concerns expressed by barramundi farmers resulted in the investigation of the interaction between larval barramundi and various life stages of *A. dengizicus* in Chapter 3

(Section 3.2.3) following the documentation of the life cycle of the cyclopoid (Section 3.2.1) and quantification of culture conditions (Section 3.2.2).

Table B4.1: Copepod genera identified from zooplankton samples collected from ponds on Reid's Barramundi Farms NT during the month of November 1995.

Order	Genus	Reported use in aquaculture
Calanoida	*Pseudodiaptomus* spp	Doi et al., 1997c ;Toledo et al. 1999; Hagiwara et al., 2001;
Cyclopoida	*Apocyclops dengizicus*	Indulkar et al., 1994
	Oithona sp	Lokman, 1993; Singhagraiwan & Doi, 1993; Toledo et al. 1999;
Harpacticoida	*Euterpina acutifrons*	Kraul, 1983; Kraul, 1989; Kraul et al., 1991

Appendix C
Acartia

C1 Introduction

The commencement of work on *Acartia* required attention be directed toward a number of additional factors. Unlike the harpacticoid *Tisbe* and the cyclopoid *Apocyclops*, the calanoid *Acartia* demonstrated a low tolerance of handling, and a requirement for more stringent culture parameters in terms of the range of salinity, light intensity and culture volume supportive of population density increases.

In the process of undertaking a number of experiments with *Acartia* it became apparent that the absence of external egg sacs complicated the selection of inoculum as the greater level of handling required to determine the gender of *Acartia* was associated with reduced survival of the selected copepods.

The experiments detailed in this Appendix represent preliminary investigations into:

- the most appropriate life stage with which to inoculate trials (C2),
- the effect of two levels of natural versus artificial light on Acartia culture performance (C3),
- the potential of a local rhodomonad microalgal species as a suitable diet for Acartia (C4), and
- the suitability of 10 L culture volumes experiencing 95 % volume water exchange at an eight-day interval for the assessment of diet on Acartia culture population performance (C6).

Difficulties experienced when using golden snapper as a test species in small volume aquaria resulted in attempts to answer questions relating to the suitability of *Acartia* as a live food using barramundi. *A. plumosa,* dominant in the seawater ponds in West Java, has been collected and used successfully in the larval rearing of barramundi and grouper (Sunyoto et al., 1995). Subsequently preliminary trials were conducted to assess the relative performance of barramundi larvae fed *Acartia* nauplii as an alternative live food either in isolation or as a component of a mixed diet comprising rotifers and *Artemia* (C7).

C2 Assessment of *Acartia* eggs and nauplii as inoculum for factorial temperature by salinity trial

Acartia being a broadcast spawner literature indicated that eggs should provide a suitable life stage for inoculation on the basis that they are readily separated from other life stages as they are negatively buoyant, and those collected within a specified time frame (Kimmerer & McKinnon, 1987; Støttrup & Jensen, 1990; Sunyoto et al., 1995).

The following trial was specifically designed to assess the effect of salinity and temperature over the ranges 5 ‰ to 45 ‰ and 23 °C to 35 °C on *Acartia* performance. However, difficulties experienced in obtaining sufficient quality eggs with which to inoculate trials resulted in the investigation of alternative life stages to use as inocula.

C2.1. Materials and methods

Ten 100 L water baths were configured and calibrated as described in Section 2.2.2 one week prior to the inoculation of the experiment. Two water baths were set to each of the treatment temperatures 23 °C, 26 °C, 29 °C, 32 °C and 25 °C. The ten water baths were arranged in two rows of five, corresponding to blocks, in a constant temperature room

maintained at 22 ±2 °C. Water bath temperatures were monitored twice daily and adjusted where necessary.

Culture media were made up in batches using a combination of Aquasonic® Ocean Nature artificial sea salt, acclimated tap water and algae to achieve the seven treatment salinities: 5 ‰, 15 ‰, 20 ‰, 25 ‰, 30 ‰, 35 ‰ and 45 ‰. All culture media contained *Isochrysis* at $1x10^5$ cells mL^{-1} and *Tetraselmis* at $6x10^4$ cells mL^{-1}. Nitrite and ammonia levels of the fresh media were recorded prior to the distribution of 200 mL volumes to each of the seventy culture units.

A 75% culture medium exchange was conducted every third day, the spent culture medium was siphoned out through a 44 µm mesh screen to prevent the removal of any copepod individuals and replaced with freshly prepared, temperature acclimated culture media of the appropriate salinity. The salinity, pH and DO levels of the spent media were recorded, and the dominant copepod life stage present in the culture unit noted. Ammonium and nitrite levels were also monitored in spent media obtained from two replicates maintained at each treatment temperature.

Inoculum A

Acartia copepodids collected from Vesty's Lake, cleaned in the 'plankton washer' as previously described in Section 3.2.2, and concentrated to 10 L were transferred to the trial egg culture system (Figure C3.1) and maintained on a light *Tetraselmis* bloom (~$1x10^4$ cells mL^{-1}).

In excess of 1,400 eggs were collected from the bottom bucket of the dedicated egg collection culture system and provided with fresh culture media at 30 ‰ with a light mixture of *Tetraselmis* and *Isochrysis*. Twenty nauplii, hatching from the collected eggs were randomly distributed to each of 70 plastic vessels containing 10 mL of fresh seawater at 30 ‰. Prior to the inoculation of the 200 mL culture units, each of the 10 mL inoculation media were partly adjusted to the appropriate treatment salinity over a two hour period by the addition of two 3 mL volumes of media at the appropriate salinity. Following a period of temperature acclimation, ten replicate culture units at the seven salinities were inoculated with nauplii adjusted to the appropriate salinity and duplicate cultures for each salinity distributed to the five treatment temperatures.

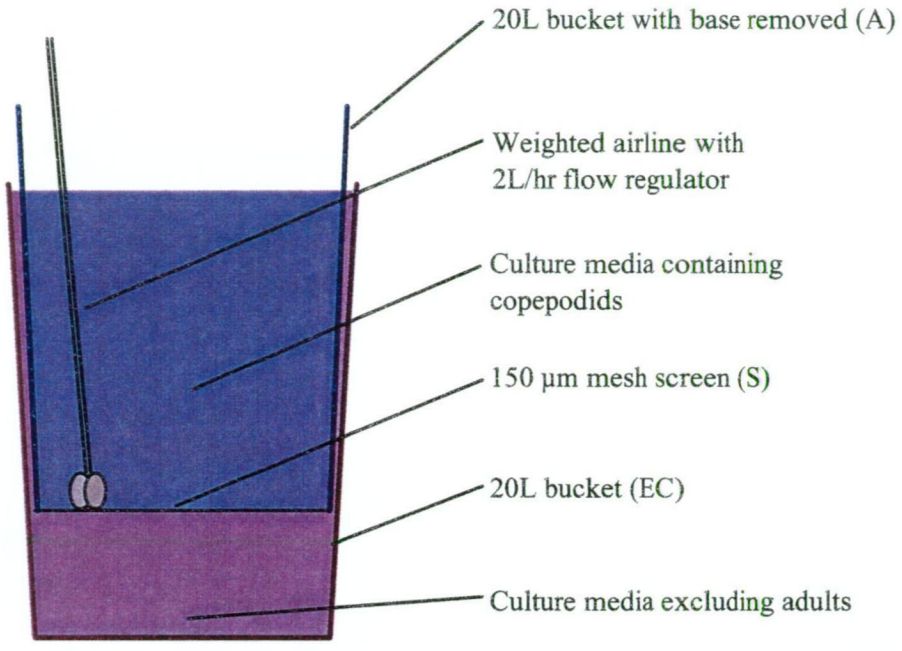


Figure C3.1: Trial *Acartia* egg collection culture system. Consisting of two 20 L buckets, one of which retained the 15 L culture media (EC), the other retained the adult *Acartia* copepodids (A). The second bucket had the base removed and replaced with a 150 µm mesh screen (S) which retained the copepodids, but allowed eggs broadcast by adult *Acartia* to pass through the screen and settle on the bottom of the 20 L bucket (EC). The culture system was fitted with a weighted airline delivering air at ~2L hour^{-1} to maintain homogeneity of the culture media.

Inoculum B

After two days a 200 µm-mesh screen was pushed down through the culture, removing adults from the upper region of the culture. The density of the separated nauplii were then determined by counting three random samples under a dissecting microscope prior to inoculation of experimental units at a density equivalent to 300 nauplii L^{-1}. Insufficient nauplii were recovered.

Subsequently *Acartia* copepodid stages developing from the culture described above were maintained in a lightly aerated mixture of *Tetraselmis* and *Isochrysis* under natural light and temperature conditions. After four days the *Acartia* culture was concentrated and 1400 copepodids transferred from the concentrated stock culture to 70 mL vessels containing 10 mL of fresh seawater at 30 ‰ using a 20 mL beaker.

Prior to the inoculation of the 200 mL culture units, each of the 70 mL inoculum cultures containing 20 copepodids were partly adjusted to the appropriate treatment salinity over a two hour period by the addition of two 10 mL volumes of media at the appropriate salinity. Following a period of temperature acclimation, ten replicate culture units at the seven salinities were inoculated with copepodids adjusted to the appropriate salinity and duplicate cultures for each salinity distributed to the five treatment temperatures.

Sample Analyses

At the end of the trial, the contents of each culture unit was screened down to a volume of 20 mL and 4 mL of a 1:1 formalin glycerol preservative added. Final salinity, pH and DO levels were recorded for each replicate, with ammonium and nitrite levels recorded for four replicates at each treatment temperature, two from each block.

The number of females, males, late copepodids (CIV and CV), early stage copepodids (CI-CIII), late nauplii (NIV-NVI), early nauplii (NI-NIII) and eggs were enumerated using an Olympus SZ40 stereo dissecting microscope.

C2.2. Results

Inoculum A

Difficulties were experienced using *Acartia* eggs as the inoculation life stage. Obtaining sufficient eggs to achieve a reasonable inoculum density combined with inconsistent and unpredictable hatch rate of *Acartia* eggs confounded any results possibly obtained. Consequently the second run of the trial (Inoculum B) was inoculated using *Acartia* copepodids.

Inoculum B

The ten *Acartia* copepodids used as an inoculum for each 200 mL culture also failed to yield a significant number of survivors after the initial three days prior to the first scheduled water exchange. A 75% water exchange was conducted and the culture units were left for a further 3 days, after which time the pots were siphoned down to 30 mL through a 44 µm mesh screen, and the culture media retained checked for surviving *Acartia*. Few, if any, copepodid stages were evident. The culture units were refilled and left at their respective treatment salinity and temperature combinations for a further three days prior to documenting the number of persistent *Acartia*. Due to the small number of persistent copepod stages, presence or absence data only was collected (Table C3.1) as insufficient data was collected with which to conduct formal statistical analyses.

No representatives from any of the three life stage categories – copepodids (CI through CVI), nauplii (NI through NVI) or eggs - were persistent in any of the cultures at 5 ‰ across all temperatures. Cultures maintained at 35 ‰ supported the full complement of life stages across all temperatures. It would appear 35 ‰ is the preferred salinity.

Temperature over the range investigated (23 to 35 °C) does not appear to exert a strong influence on *Acartia* culture productivity.

C2.3. Discussion

Salinity was observed to exert a strong influence on *Acartia* culture productivity, temperature effects being almost non-existent in comparison. *Acartia* numbers were significantly greater at 35 ‰ across all temperatures, with eggs more numerous at lower temperatures most probably as a result of slowed development.

Pagano & Saint-Jean (1994) reported temperature and salinity to have no effect on the fecundity of tropical *A. clausi*. Recognising the strong influence of salinity on the population of the Australian *Acartia*, it is reasonable to suggest that the species is a coastal not brackish water species. The narrow salinity range supporting *Acartia* productivity also explains the observed seasonality of the species in the coastal lagoon from which it was collected (Glenn Schipp, DAC, *pers. comm.*).

Table C3.1: Presence (✓) or absence (✘) of *Acartia* copepod stages persisting in duplicate 200 mL cultures across seven salinities (5, 15, 20, 25, 30, 35 & 45 ‰) and five temperatures (23, 26, 29, 32 & 35 °C) after nine days. Where duplicate cultures differed in response to the culture conditions, two symbols are presented.

T °C	S‰	Copepodids	Nauplii	Eggs
23	5	✘	✘	✘
26	5	✘	✘	✘
29	5	✘	✘	✘
32	5	✘	✘	✘
35	5	✘	✘	✘
23	15	✓	✘	✘
26	15	✘/✓	✘	✘
29	15	✘	✘	✘
32	15	✘/✓	✘	✘
35	15	✓	✘	✘
23	20	✓	✘	✘
26	20	✓	✓	✓
29	20	✓	✘	✘
32	20	✓	✘/✓	✘/✓
35	20	✓	✘/✓	✘/✓
23	25	✓	✓	✘
26	25	✓	✓	✓
29	25	✓	✓	✘/✓
32	25	✓	✘	✘
35	25	✓	✘	✓
23	30	✓	✓	✓
26	30	✓	✓	✓
29	30	✓	✘/✓	✘/✓
32	30	✓	✘/✓	✘/✓
35	30	✘/✓	✘	✘
23	35	✓	✓	✓
26	35	✓	✓	✓
29	35	✓	✓	✓
32	35	✓	✓	✓
35	35	✓	✓	✓
23	45	✓	✘	✘
26	45	✓	✘/✓	✓
29	45	✓	✘/✓	✘/✓
32	45	✓	✓	✓
35	45	✓	✘/✓	✓

The implications of the difficulties associated with collecting *Acartia* eggs and nauplii has implications for future trials. The most easily obtained life stage would appear to be the later stage copepodids. The subitaneous eggs of the Australian *Acartia* would appear also to possess spines on the outer shell (Knuckey et al., 2001; Gail Semmens, QDPI, *pers. comm.*) which render them 'sticky' and difficult to separate from the algal mat which developed in the 20 L egg collection culture system.

C3 The influence of light source and culture volume on the performance *Acartia* in culture

While conducting trials assessing the influence of temperature and salinity on *Acartia* productivity, it was evident that the light conditions imposed and culture volume used were not suited to the persistence of *Acartia* populations in culture. In order to determine which aspect of the culture environment was limiting, trials were conducted to assess the influence of light level and culture volume on *Acartia* productivity as indicated by culture population density.

C3.1. Materials and methods

Two 100 L water baths were configured to accommodate six 150 mL and six 25 mL floating culture vessels. One water bath was located inside the constant temperature room with an incandescent source. The second 100 L water bath was floated in a 7 m^3 tank exposed to natural sunlight experiencing an average light intensity of 6000 lux.

Culture media was made up in a single 7L volume of 1 µm filtered ultra-violet treated seawater at 35 ‰ to which was added *Isochrysis* at $3.3x10^4$ cells mL^{-1}, *Rhodomonas* at $2x10^4$ cells mL^{-1} and *Tetraselmis* at $2x10^4$ cells mL^{-1}. Prior to treatment volumes being dispensed the temperature, pH, salinity and levels of nitrogenous compounds (unionised ammonia and mg nitrite L^{-1}) were checked using the equipment and methods detailed in Section 4.2.

The *Acartia* used to inoculate the trial were collected after dark using light as a concentrating tool from the 100 L stock cultures maintained as described in Section 4.2.2. The concentrated *Acartia* copepodids were transferred to culture units using a large bore plastic pipette (Ø 3 mm).

Six 25 mL cultures inoculated with 3 *Acartia* copepodids were maintained under artificial light conditions, and complementary six maintained under natural light conditions. Five 150 mL cultures inoculated with 18 *Acartia* were maintained under artificial light conditions, and a complementary five maintained under natural light conditions. The inoculation density used corresponds to a density equivalent to 120 copepodids L^{-1} which is intermediate between 80 and 160 copepodids per L^{-1}, identified as optimal inoculation range in Section 4.3.2.2. Once inoculated the culture units were placed in water baths.

The *Acartia* cultures were left for five days with water temperature monitored daily. Outdoor light levels were recorded mid-morning and mid-afternoon, and the general health of all *Acartia* populations observed by lifting the cultures from their supportive skirts, holding them up to the light and observing the activity and number of life stages present. After 5 days, environmental parameter data was recorded and the contents of all culture units were preserved with the addition of a 5% solution of formalin and glycerol.

Sample & Statistical Analysis

The number of females, males, late copepodids (CIV and CV), early stage copepodids (CI-CIII), late nauplii (NIV-NVI), early nauplii (NI-NIII) and eggs were enumerated using an Olympus SZ40 stereo dissecting microscope.

All environmental parameter and *Acartia* data collected were subjected to Shapiro-Wilks test for normality and Bartlett's test for homogeneity of variance. Treatment effects in normal data exhibiting homogenous variance were determined by analysis of variance (ANOVA) and Scheffe's multiple means comparison tests. Data unable to be transformed to meet the assumptions of ANOVA were analysed either using Kruskal-Wallis *k*-sample test or Mann-Whitney *U*-test in conjunction with Tukey's multiple means comparison test.

Culture population density data are reported as the equivalent number of individual *Acartia* L^{-1} to enable comparison between trials conducted in culture units of different volumes.

C3.2. Results

Light source and culture volume exerted significant influences on the survival and productivity of *Acartia* copepodids. The total number of *Acartia* persisting in the 25 mL and 150 mL cultures under the different light conditions was shown to be significantly different by Kruskal-Wallis *k*-sample test ($p<0.05$, Figure C3.1). The 150 mL cultures maintained under artificial light conditions yielded densities equivalent to 200 *Acartia* L^{-1}, ten times greater than in cultures maintained under natural light conditions, or in 25 mL volumes.

Acartia 150 mL cultures exposed to artificial light produced an average 600 eggs L^{-1} compared with the 13 eggs L^{-1} yielded by 150 mL cultures maintained under natural light conditions for five days (Figure C3.2). Only one 25 mL culture maintained under artificial light produced one egg.

No nauplii were seen in the 25 mL or 150 mL *Acartia* cultures maintained under natural light compared to the 200 nauplii L^{-1} hatching in 150 mL cultures maintained under artificial light conditions.

The statistical significance and mean values for 150 mL *Acartia* cultures maintained under the two light conditions are detailed in Table C3.1.

Throughout the trial, microalgal cells were visible in suspension, with algae more dense and green in both the 25 mL and 150 mL cultures maintained under natural light conditions. Cultures maintained under natural light conditions experienced greater light intensity, slightly higher culture temperature and elevated salinity and dissolved oxygen levels compared to those maintained under artificial light conditions (Table C3.2). Nitrogenous compounds were below detectable limits in all culture media prior to inoculation with *Acartia*, and after 5 days of culture under all treatment light conditions.

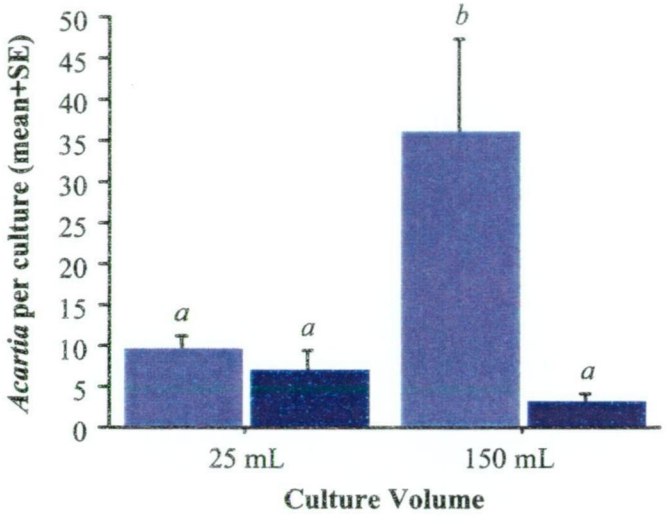


Figure C3.1: The influence of culture volume (25 and 150 mL) and light source (■ artificial and ■ natural) on *Acartia* culture productivity when maintained on a mixed algal diet. Italicised superscripts indicate significant differences ($p<0.05$) identified by Kruskal-Wallis *k*-sample analysis and Tukey's multiple means comparison.

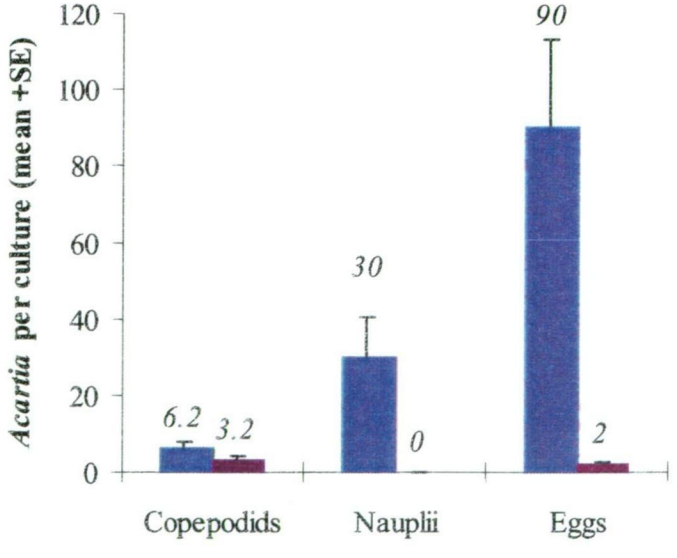


Figure C3.2: *Acartia* life stage distribution in 150 mL volume cultures maintained under ■ artifiical and ■ natural light conditions.

Table C3.1: Demographic summary of *Acartia* populations maintained under either artificial or natural light conditions in 150 mL cultures fed a mixed algal diet of *Isochrysis*, *Rhodomonas* and *Tetraselmis* resulting in a final cell density of 7.3 x10^4 cells mL^{-1}.

	Artificial (mean ±SE)	Natural (mean ±SE)	*p*-value
Total individuals L^{-1}	240 ±74	21.3 ±6.5	<0.05
Adults L^{-1}	41 ±10.6	20 ±5.2	>0.05
Nauplii L^{-1}	200 ±67	0	<0.05
Eggs L^{-1}	600 ±153	13 ±4.7	<0.05
Sex ratio (F:M)	1.0 ±0.1	1.8 ±1.1	>0.05

Table C3.2: Average environmental parameters recorded for *Acartia* cultures maintained under the two light conditions. (n/m – not measured; nd – not detected). Initial refers to culture media prior to distribution to the culture units, Artificial and Natural corresponding to treatment media after six days.

Parameter	Initial	Artificial	Natural
Temperature (°C)	26	30.1 ±0.16	32.7 ±0.67
Salinity (‰)	35	34.4 ±0.35	35.4 ±0.19
pH	8.23	8.0 ±0.05	9.7 ±0.16
Light (lux)	n/m	600 ±60	5 600 ±410
DO (mgO_2 L^{-1})	7.8	7.2 ±0.23	11.6 ±0.55
DO (% O_2)	98	96 ±3.3	160 ±6.5

C3.3. Discussion

The results obtained served to highlight the requirement for shading of outside cultures of *Acartia*, as the direct sunlight subjected the algae and copepods to extreme temperatures during the middle of the day. Marshall (1973) reported full sunlight to be lethal to the calanoid *Calanus finmarchicus* due to increased oxygen consumption and direct damage to the animals. The high light intensity combined with the elevated temperatures and high pH levels may well have been to the detriment of the outside copepod cultures maintained under natural light conditions.

The cultures located inside under artificial light conditions proved more productive than the outdoor cultures, although a high percentage of eggs remained unhatched, possibly due to the time of sampling or the effect of lower light intensities. Landry (1975) observed nauplius hatching of *Acartia clausi* to be inhibited under constant darkness.

The trial did not include a control in the form of culture conditions maintained under normal light conditions without supplemental lighting, nor did it include a greater volume than 150 mL due to a lack of available inoculum. The investigation detailed in section 4.2.2.1. into the influence of light and volume on *Acartia* culture was designed to address these shortcomings.

C4 Preliminary assessment of a Northern Territory isolate of the microalgae *Rhodomonas* as a diet for *Acartia*

In conjunction with temperature and salinity, diet is the most easily manipulated culture condition, and also one of the most important from the perspective of potential for growth and development of copepod life stages (McKinnon, 1996).

Acartia species are primarily algal feeders (Allan, 1976; Norsker & Støttrup, 1994). The following trial designed to assess the relative potential of a Darwin Harbour isolate of the microalgae *Rhodomonas* to the culture of the Darwin species of *Acartia*. The local *Rhodomonas* isolate exhibited the desirable characteristics of appropriate size falling within the range 10-15 µm identified by Berggreen et al. (1988), a motile cell able to remain in suspension and available to filter feeding organism with minimal aeration, and good growth under local tropical conditions.

C4.1 Materials and methods

Algal Culture Maintenance

The microalgae used in the following trials included the local Northern Territory isolate of *Rhodomonas* and the CSIRO isolates of *Isochrysis* and *Tetraselmis* and maintained under the same culture conditions as described in the Common Methods in Chapter 3 Section 3.2.

Experimental Protocol

The *Acartia* copepodids used as the inoculum for this short-term trial were collected the night before the trial from Vesty's Lake as described in Chapter 4 Section 4.3.2.

The seven treatment diets detailed in Table C4.1 were prepared using axenic algal cultures in combination with 0.1 µm ultraviolet filtered seawater at 30 ‰.

Table C4.1: Details of the cell densities for the algal species combinations corresponding to the seven diets assessed for the maintenance of *Acartia* populations. Iso – *Isochrysis*, Rho – *Rhodomonas* and Tet – *Tetraselmis*.

Diet	*Isochrysis* (cells mL^{-1})	*Rhodomonas* (cells mL^{-1})	*Tetraselmis* (cells mL^{-1})
Iso	$1x10^5$	0	0
Rho	0	$6x10^4$	0
Tet	0	0	$6x10^4$
Iso & Rho	$5x10^4$	$3x10^4$	0
Rho & Tet	0	$3x10^4$	$3x10^4$
Iso & Tet	$5x10^4$	0	$3x10^4$
Rho, Tet & Iso	$3x10^4$	$2x10^4$	$2x10^4$

Three replicate 500 mL culture units were filled with each of the seven diets and inoculated with 20 *Acartia* copepodids (40 *Acartia* L^{-1} previously identified as the minimum inoculation density required to achieve reliable results) ranging from CII to CVI.

The twenty-one culture units were distributed between three water baths maintained at 29 °C in a constant temperature room exposed to 300 lux and 13L:11D photoperiod, and left untouched for three days.

An 80% culture medium exchange was conducted on the third day with concurrent collection of data on temperature, salinity, pH, dissolved oxygen and a qualitative assessment of culture health.

The trial was terminated on day 6 at which time all pots were siphoned down to 50 mL and 10 mL of 1:1 formalin glycerol mixture added.

Sample & Statistical Analysis

Samples and subsequent data were treated in the same manner as described above in B3.1.

C4.2 Results

The total number of *Acartia* developing in culture over the three days of the trial generally indicates population increases with the number of species of microalgae used in preparation of the diets, although no significant difference was identified ($p>0.05$). The maximum density achieved was equivalent to 710 *Acartia* L^{-1} from cultures fed a mixed microalgal species diet. A maximum of 45 eggs L^{-1} were recovered. The average number of *Acartia* developing over the three days of the trial was 80 ±10 individuals L^{-1}. *Rhodomonas* based diets yielded consistently productive cultures. Successful reproduction was observed in all cultures irrespective of diet composition.

The number of eggs and copepodids recovered from the cultures did not show any significant differences between algal species averaging 184 eggs L^{-1} (maximum 710 L^{-1} from the mixed diet) and 26 copepodids L^{-1} (maximum of 45 L^{-1} also from cultures fed the algal mixture).

Naupliar numbers on the other hand differed significantly ($p<0.05$) between treatments (Figure C4.1). Diets containing *Rhodomonas* appear to produce a greater number of individuals, with mixed diets resulting in the greatest density of *Acartia* (Table C4.2).

Table C4.2: The average density of *Acartia* life stages (mean ±standard error) present in cultures maintained on different algae at 29°C and 35 ‰ for three days. I – *Isochrysis*, R – *Rhodomonas*, T – *Tetraselmis*.

Diet	Total L^{-1}	Eggs L^{-1}	Nauplii L^{-1}	Copepodids L^{-1}
Isochrysis	57 ±9.7	184 ±52	26 ±3.7	31 ±7.8
Rhodomonas	69 ±11	143 ±58	44 ±8.5	25 ±5.2
Tetraselmis	26 ±1.5	278 ±38	14 ±2.8	12 ±1.5
I+R	67 ±16	213 ±27	44 ±11.5	23 ±6.6
I+T	60 ±8.7	297 ±51	35 ±8.7	25 ±2.5
R+T	124 ±15	334 ±77	93 ±16	31 ±3.8
I+R+T	148 ±49	361 ±174	113 ±43	34 ±5.0

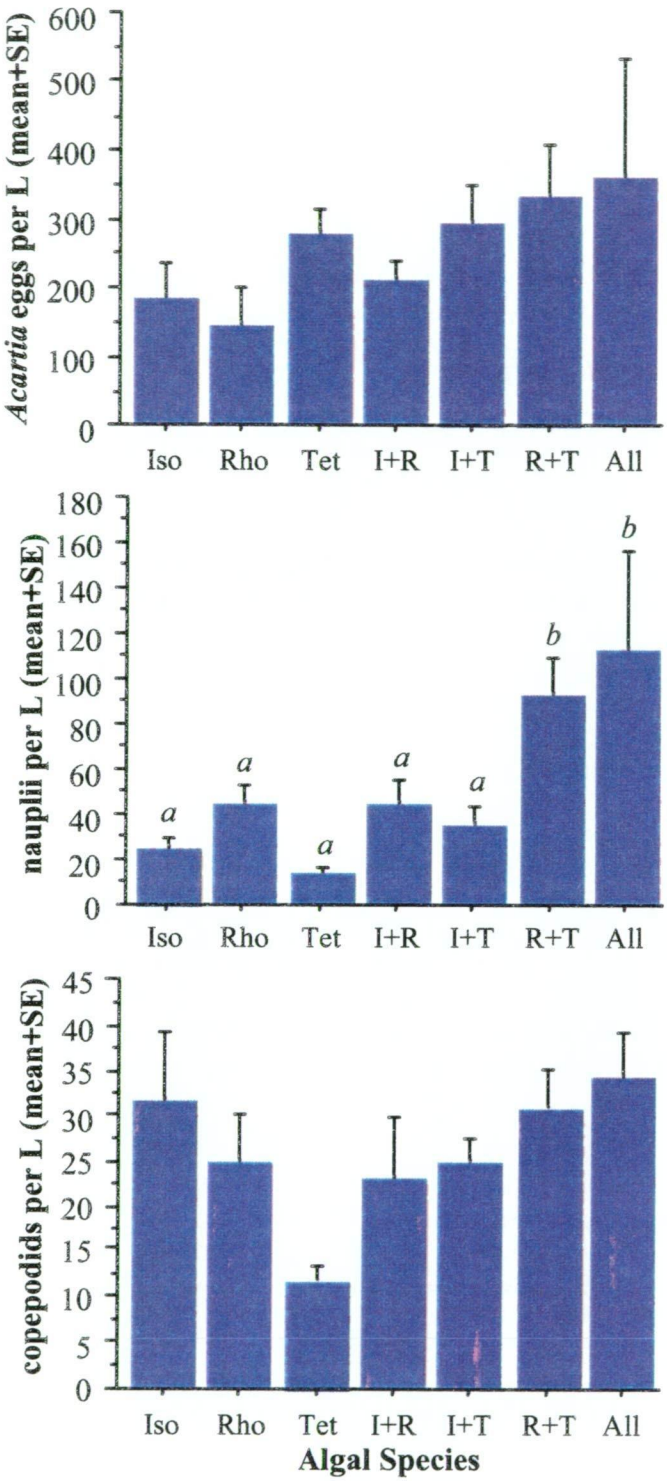


Figure C4.1: The influence of diet on the number of *Acartia* nauplii, copepodids and eggs developing after 3 days when maintained at 29 °C and a salinity of 30 ‰. Italicised superscripts indicate significant differences revealed by ANOVA of square root transformed data and identified by Scheffe's multiple means comparison test. Iso, I – *Isochrysis*, Rho, R – *Rhodomonas*, Tet, T – *Tetraselmis*, All – all three algal species each represented by one third of the total cell density offered.

C4.3 Discussion

The *Acartia* cultures fed the mixed species microalgal diet were the most dense and produced the greatest number of each of the life stage groups: eggs, nauplii and copepodids (Table C4.2). *Acartia* cultures fed *Rhodomonas* either on its own or as a component of a mixed species diet produced the most dense culture populations. The preliminary results obtained here suggest that *Rhodomonas* is a species worth further investigation, and demonstrates similar responses in copepod cultures as have been observed for Northern Hemisphere congeners of both *Acartia* and *Rhodomonas*.

Acartia presented a diet of *Isochrysis* performed similarly, if not better than those presented *Tetraselmis* in contrast to the findings of Parrish & Wilson (1978). Støttrup & Jensen (1990) also attributed the poor fate of *Acartia* with Wilson & Parrish to the low *Isochrysis* cell concentration used (5.7 x10^3 cell mL^{-1} compared with 10^5).

The 3-day duration of the trial was sufficient to detect initial responses to the microalgal diets as all the copepodids used as inocula possessed the same history and had been purged for a period of at least 12 hours. Saiz et al. (1997) found that over a 24 hour period the immediate source of food to be reflected in egg production by temperate copepod species. A number of other studies have found egg production to mirror food availability within 24 hours at 15 to 18 °C (Laabir et al., 1995; Rodríguez et al., 1995; Calbet & Alcaraz, 1996). Tester & Turner (1990) found that ingested carbon could be converted into egg carbon in as little as 6 hours at temperatures greater then 22 °C. Copepod egg quality may thus be considered as linked to the recent past feeding histories of females in their natural environment (McKinnon, 1996).

Differences between diets in terms of *Acartia* densities may be more apparent if the inoculum comprised 100% fully mature adults. The number of copepodids recovered after three days were less than that inoculated for all cultures. *Tetraselmis*-fed cultures yielding copepodid densities half that recovered from the cultures fed other diet treatments.

C5 Determination of algal species cell carbon concentration

Acartia cultures appear to exhibit greatest productivity when maintained on algal densities corresponding to 500 µgC L^{-1} (Robert Campbell, University of Rhode Island, *pers comm.*). Eppley et al. (1970) devised a convenient method by which to estimate algal cell carbon content indirectly without resorting to high temperature combustion (Smayda, 1978). Algal cell carbon content was achieved using estimated cell volume for each algal species cell type in conjunction with the accepted relationship described by the formulae described by Eppley et al. (1970):

$C = \text{inverse } \log_{10} (0.76 \log V - 0.352)$ for diatoms

$C = \text{inverse } \log_{10} (0.94 \log V - 0.6)$ for other phytoplankton

where C is pg carbon $cell^{-1}$, and V is the mean estimated cell volume calculated from direct measurement of algal cell dimensions.

5.1 Materials and methods

A random sample of algal cells was obtained from axenic cultures of the five microalgae *Cryptomonas*, *Heterocapsa*, *Isochrysis*, *Nitzschia*, *Rhodomonas* and *Tetraselmis* reaching the end of their exponential growth phase at densities similar to those required for the completion of feeding trials. The microalgae used were those described in the Common Methods Chapter 3 Section 3.2 maintained under the same culture conditions. Algal cells were mounted on slides, cover-slipped and the dimensions of fifteen individual cells

measured using a calibrated ocular graticule at 400x magnification on an Olympus compound microscope. The appropriate length and width (or diameter) measurements were converted from graticule units to microns (µm) using the appropriate conversion factor (CF = 2.5) determined using a stage mounted micrometer.

Approximate cell volumes were calculated using two equations:

$V = \pi r^2 l$ for cylindrical cells

$V = {}^4/_3 \pi r^2$ for spherical cells

where 'r' is the cell radius taken as half the diameter measured as the widest point along the shortest axis, and 'l' being the cell length measured on longest axis

The mean values for each of the step-wise calculations are summarised in Tables B5.1 and B5.2.

The density of each microalgae corresponding to a carbon cell concentration of 500 µgC L^{-1} were then calculated:

Algal Density (cells mL^{-1}) = Carbon Concentration (ngC mL^{-1}) / Algal Cell Carbon (pg C $cell^{-1}$)

where Carbon Concentration is the desired carbon per volume required, i.e. $5x10^5$ ngC mL^{-1} equivalent to 500 µgC L^{-1}.

5.2 Results

The series of calculations yielded desired densities of $5.7x10^4$ cells mL^{-1} for *Tetraselmis*, $6.8x10^4$ cells mL^{-1} *Rhodomonas*, and $2.0x10^5$ cells mL^{-1} for *Isochrysis*, $1.4x10^5$ cells mL^{-1} for *Cryptomonas* and $1.7x10^5$ cells mL^{-1} for *Nitzschia*. The values presented in Table C5.3 are the figures first calculated on which all subsequent ration determinations were based. Raw data from which values in Tables C5.1 and C5.2 were derived are presented in Tables C5.3 through C5.8.

Table C5.1: Mean cell length, cell diameter and cell volume (mean ±standard error) for the six microalgae as determined by microscopic measurement.

	Shape	Length (µm)	Diameter (µm)	Volume ($µm^3$)
Cryptomonas	Cylinder	7.4 ±0.28	4.2 ±0.21	104 ±9.17
Heterocapsa	Sphere + Cylinder	19.6 ±0.46	12.0 ±0.28	1776 ±111
Isochrysis	Sphere	-	5.8 ±0.15	104 ±8.0
Nitzschia	Cylinder	17.5 ±0.08	2.5 ±0.00	86 ±0.4
Rhodomonas	Cylinder	9.2 ±0.28	5.6 ±0.17	230 ±20
Tetraselmis	Cylinder	13.4 ±0.26	8.4 ±0.26	765 ±67

Table C5.2: Mean values (±standard error) calculated for algal species cell carbon content and appropriate cell density (cells mL^{-1}) required achieve a ration equivalent to 500 µgC L^{-1} and 1000 µgC L^{-1}.

	pgC $cell^{-1}$	500µg L^{-1}	1000µg L^{-1}
Cryptomonas	3.6 ±0.53	1.4×10^5	2.8×10^5
Heterocapsa	234 ±6.2	2.1×10^3	4.3×10^3
Isochrysis	2.5 ±0.55	2.0×10^5	4.0×10^5
Nitzschia	2.9 ±0.00	2.6×10^5	5.2×10^5
Rhodomonas	7.3 ±1.11	6.8×10^4	1.4×10^5
Tetraselmis	8.7 ±1.30	5.7×10^5	1.1×10^5

5.3 Discussion

The algal cell densities calculated were subsequently used to prepare mixed species microalgal diets exhibiting the same cell carbon concentration ration.

Table C5.3: Raw data for *Cryptomonas* (Northern Territory University collection CRFI01) cell carbon content.

Cell	Length (µm)	Diameter (µm)	Cylinder (μm^3)	log C	pg C per cell
1	5.5	5.0	108	1.31	20.5
2	8.5	3.0	60	1.07	11.8
3	7.5	5.0	147	1.44	27.4
4	10.0	3.0	71	1.14	13.8
5	7.5	3.3	62	1.09	12.2
6	7.5	5.0	147	1.44	27.4
7	7.5	4.0	94	1.26	18.0
8	6.0	4.0	75	1.16	14.6
9	7.0	5.0	137	1.41	25.7
10	8.0	4.8	142	1.42	26.5
11	7.5	3.3	62	1.09	12.2
12	7.5	5.0	147	1.44	27.4
13	7.5	4.0	94	1.26	18.0
14	6.0	4.0	75	1.16	14.6
15	7.0	5.0	137	1.41	25.7
mean	7.4	4.2	104	1.27	19.7
SE	0.28	0.21	9.17	0.04	1.64

Table C5.4: Raw data for *Heterocapsa* (CSIRO collection CS36) cell carbon content.

Cell	Length (µm)	Diameter (µm)	Sphere ($µm^3$)	cylinder ($µm^3$)	Total ($µm^3$)	log C	pgC per cell
1	20.0	11.5	796	883	1679	2.10	125.6
2	20.0	13.0	1150	929	2079	2.17	147.8
3	20.5	14.5	1596	991	2587	2.24	174.5
4	20.0	11.5	796	883	1679	2.10	125.6
5	21.0	13.0	1150	1062	2212	2.19	154.9
6	18.0	11.5	796	675	1471	2.06	113.6
7	17.0	12.0	905	565	1470	2.06	113.6
8	20.0	12.4	998	918	1916	2.14	138.9
9	19.0	12.0	905	792	1696	2.10	126.6
10	19.0	11.5	796	779	1575	2.08	119.7
11	23.5	13.0	1150	1394	2544	2.24	172.3
12	21.0	11.0	697	950	1647	2.09	123.8
13	20.0	11.5	796	883	1679	2.10	125.6
14	16.0	10.0	524	471	995	1.93	84.4
15	18.5	11.0	697	713	1410	2.04	110.0
mean	19.6	12.0	916.9	859.2	1776	2.11	130.5
SE	0.46	0.28	67.47	56.29	110.7	0.02	6.18

Table C5.5: Raw data for *Isochrysis* (CSIRO collection CS177) cell carbon content.

Cell	Diameter (µm)	Sphere ($µm^3$)	log C	pg C per cell
1	6.5	144	1.43	26.8
2	6.0	113	1.33	21.4
3	5.0	65	1.11	12.8
4	5.0	65	1.11	12.8
5	6.5	144	1.43	26.8
6	6.0	113	1.33	21.4
7	7.0	180	1.52	33.0
8	6.0	113	1.33	21.4
9	5.5	87	1.22	16.7
10	6.0	113	1.33	21.4
11	5.5	87	1.22	16.7
12	6.0	113	1.33	21.4
13	5.0	65	1.11	12.8
14	5.5	87	1.22	16.7
15	5.0	65	1.11	12.8
mean	5.8	103.7	1.28	19.7
SE	0.15	7.99	0.03	1.42

Table C5.6: Raw data for *Nitzschia* (Northern Territory University collection NT7) cell carbon content.

Cell	Length (µm)	Diameter (µm)	Cylinder ($µm^3$)	log C	pg C per cell
1	17.5	2.5	86	1.12	13.1
2	18.0	2.5	88	1.13	13.4
3	17.5	2.5	86	1.12	13.1
4	17.0	2.5	83	1.11	12.8
5	17.5	2.5	86	1.12	13.1
6	17.5	2.5	86	1.12	13.1
7	17.5	2.5	86	1.12	13.1
8	17.0	2.5	83	1.11	12.8
9	17.5	2.5	86	1.12	13.1
10	17.5	2.5	86	1.12	13.1
11	17.0	2.5	83	1.11	12.8
12	17.5	2.5	86	1.12	13.1
13	17.5	2.5	86	1.12	13.1
14	18.0	2.5	88	1.13	13.4
15	17.5	2.5	86	1.12	13.1
mean	17.5	2.5	86	1.12	13.1
SE	0.08	0.00	0.38	0.00	0.04

Table C5.7: Raw data for *Rhodomonas* (Northern Territory University collection NT15) cell carbon content.

Cell	Length (µm)	Diameter (µm)	Cylinder ($µm^3$)	log C	pg C per cell
1	9.0	5.5	214	1.59	38.9
2	8.5	5.0	167	1.49	30.8
3	10.0	6.0	283	1.70	50.6
4	9.0	6.0	254	1.66	45.8
5	10.0	6.0	283	1.70	50.6
6	9.0	6.0	254	1.66	45.8
7	8.0	4.5	127	1.38	23.9
8	11.0	7.0	423	1.87	74.0
9	9.0	6.0	254	1.66	45.8
10	11.0	6.0	311	1.74	55.4
11	9.0	5.0	177	1.51	32.5
12	8.0	5.0	157	1.46	29.1
13	7.0	5.0	137	1.41	25.7
14	9.0	5.0	177	1.51	32.5
15	10.0	5.5	238	1.63	43.0
mean	9.2	5.6	230	1.60	41.6
SE	0.28	0.17	20.20	0.04	3.43

Table C5.8: Raw data for *Tetraselmis* (CSIRO collection TEQL01) algal cell carbon content.

Cell	Length (µm)	Diameter (µm)	Cylinder (μm^3)	log C	pg C per cell
1	14.0	8.0	704	2.08	119.3
2	12.0	8.0	603	2.01	103.2
3	14.0	8.0	704	2.08	119.3
4	13.0	7.5	574	1.99	98.5
5	13.0	7.0	500	1.94	86.6
6	12.0	7.5	530	1.96	91.4
7	16.0	11.0	1521	2.39	246.1
8	13.0	8.5	738	2.10	124.7
9	14.0	8.5	794	2.13	133.7
10	13.0	8.5	738	2.10	124.7
11	14.5	10.0	1139	2.27	187.5
12	13.0	9.0	827	2.14	138.8
13	13.0	8.0	653	2.05	111.3
14	13.0	8.0	653	2.05	111.3
15	14.0	8.5	794	2.13	133.7
Mean	13.4	8.4	765	2.09	128.7
SE	0.26	0.26	66.8	0.03	10.45

C6 The effect of dietary algal cell carbon concentration

Temperature, salinity and diet are the most easily manipulated culture conditions. Diet is the most important from the perspective of providing the building blocks facilitating the growth and development of copepods.

Feeding and reproduction of *Acartia*, like all other copepods, is governed by the quality and abundance of available food, with size, shape and biochemical composition being important aspects of food quality (Berggreen et al., 1988; Støttrup & Jensen, 1990; Jónasdóttir, 1994; Ederington et al., 1995). The majority of literature reporting diet preferences of *Acartia* are primarily based on temperate species with little work published for tropical species.

Acartia species are recognised as omnivorous feeders capable of selective predation (Gifford & Dagg, 1988; Vanderploeg, 1994; Ederington et al., 1995), however from an aquaculture perspective they may be considered primarily filter feeders (Norsker & Støttrup, 1994).

The aim of this trial was to identify an appropriate algal cell carbon concentration promoting *Acartia* population growth. Diet composition was determined such that the total µgC L^{-1} ration was evenly distributed between the four microalgal species (*Isochrysis*, *Heterocapsa*, *Rhodomonas* and *Tetraselmis*, in media adjusted to 36 ‰ (Table C6.1 cell densities were calculated using values from Table 4.2.1 in Chapter 4).

C6.1 Materials and methods

Five replicate culture units were filled with 500 mL for each of the seven diets. The thirty-five culture units were each inoculated with 20 *Acartia* copepodids. Each 500 mL culture unit was fitted with 10L reservoir (as described in Chapter 4, Figure 4.2.1) and maintained at 30 °C with a 13L:11D photoperiod (as per diet trial in Chapter 4, Section 4.2.2.4).

Table C6.1: Algal species cell densities used to assess the effect of algal diet cell carbon concentration on *Acartia* productivity.

Concentration ($\mu gC\ L^{-1}$)	*Isochrysis* (cells L^{-1})	*Heterocapsa* (cells L^{-1})	*Rhodomonas* (cells L^{-1})	*Tetraselmis* (cells L^{-1})	Total (cells mL^{-1})
200	1.3×10^6	2.7×10^4	6.4×10^5	3.2×10^5	2.3×10^3
400	2.5×10^6	5.3×10^4	1.3×10^6	6.5×10^5	4.5×10^3
800	5.0×10^6	1.1×10^5	2.6×10^6	1.3×10^6	9.0×10^3
1,600	1.0×10^7	2.1×10^5	5.1×10^6	2.6×10^6	1.8×10^4
3,600	2.0×10^7	4.3×10^5	1.0×10^7	5.2×10^6	3.4×10^4
6,400	4.0×10^7	8.5×10^5	2.1×10^7	1.0×10^7	6.3×10^4
12,800	8.0×10^7	1.7×10^6	4.1×10^7	2.1×10^7	1.4×10^5

C6.2 Results

Acartia culture population density exhibited an inverse relationship to algal cell carbon density. No significant difference ($p>0.05$) between culture population densities was evident after six days of *Acartia* culture at algal carbon concentration over the range of 200 to 12400 $\mu gC\ L^{-1}$. The total number of *Acartia* individuals developing did however exhibit an apparent decrease at the higher algal carbon concentrations (Figure C6.1).

No significant difference was detected using ANOVA with square root transformed data, or Kruskal-Wallis *k*-sample analysis conducted with non-transformed data for the number of eggs, nauplii (NI-NVI), copepodids (CI-CVI) or sex ratio determined after six days. Life stage data exhibited a similar trend to that visible in total numbers (Table C6.2). Sex ratio did not reveal any trends. All *Acartia* populations developing at the various algal carbon concentrations yielded sex ratios less than one.

The environmental conditions prevailing across the seven algal carbon were similar ($p>0.05$; Table C6.3). All *Acartia* cultures were maintained under incident light at 1430 lux with a photoperiod regime of 13 L:11D in culture media at 35 ‰ and 31.2 °C associated with a pH of 8.2 and dissolved oxygen levels 8.4 $mgO_2\ L^{-1}$. Algal cell density was found to be significantly different between algal carbon concentrations ($p<0.01$) with each treatment level being different from all other treatments.

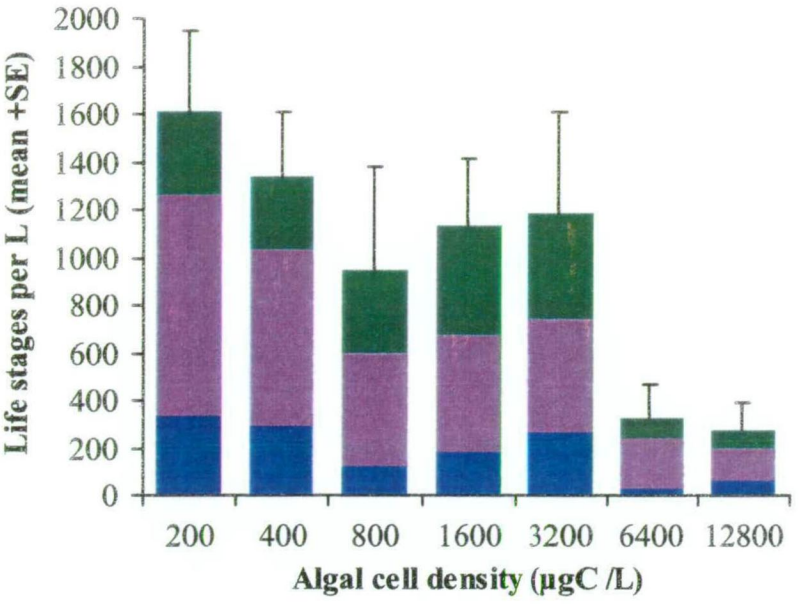


Figure C6.1: Effect of algal cell carbon concentration on *Acartia* culture productivity as determined be the number of individuals (■ copepodid stages CI through CVI, ■ nauplius stages NI through NVI and ■ eggs) developing over three days when fed a mixed algal diet consisitng of *Isochrysis*, *Heterocpasa*, *Rhodomonas* and *Tetraselmis* at 30 °C and 35 ‰.

Table C6.2: *Acartia* culture population composition (mean individuals L^{-1} ± standard error) as influenced by cell density over six days when fed a mixed algal diet consisitng of *Isochrysis*, *Heterocpasa*, *Rhodomonas* and *Tetraselmis* at 29°C and 35 ‰.

Carbon Concentration (µgC L^{-1})	Total (NI-CVI)	Copepodids (CI-CVI)	Nauplii (NI-NVI)	Eggs
200	1260 ±230	356 ±55	903 ±153	325 ±115
400	1030 ±205	315 ±76	717 ±75	290 ±130
800	800 ±425	352 ±129	445 ±250	120 ±30
1600	935 ±175	465 ±175	473 ±134	180 ±20
3200	805 ±435	334 ±229	469 ±219	260 ±20
6200	290 ±130	91 ±77	198 ±64	65 ±30
12400	195 ±125	81 ±57	114 ±71	65 ±30

Table C6.3: Summary of the environmental parameters (mean ± standard error) measured in *Acartia* culture as influenced by cell density over six days when fed a mixed algal diet consisitng of *Isochrysis*, *Heterocpasa*, *Rhodomonas* and *Tetraselmis* at 29 °C and 35 ‰.

Carbon Concentration (µg C L^{-1})	Temperature (°C)	pH	DO (mg L^{-1})	Light (lux)
200	30.2 ±0.21	8.2 ±0.05	8.0 ±1.06	1420 ±300
400	30.2 ±0.17	8.1 ±0.04	7.8 ±0.31	1380 ±160
800	30.2 ±0.30	8.1 ±0.02	8.7 ±0.32	1665 ±110
1600	30.3 ±0.09	8.2 ±0.01	9.4 ±0.78	1655 ±115
3200	30.2 ±0.14	8.2 ±0.03	8.2 ±0.59	1440 ±135
6200	30.3 ±0.13	8.2 ±0.03	8.3 ±0.60	1250 ±195
12400	30.4 ±0.20	8.2 ±0.07	8.7 ±2.14	1170 ±450

C6.3 Discussion

Cultures maintained at lower carbon concentrations were the most productive. This observation may be a reflection of the adaptation of the species to the naturally lower carbon concentrations evident in tropical marine waters, and the reduced influence of algal activity on water quality at the lower algal cell concentrations.

Ohno & Okamura (1988) found that in extensive pond culture of *A. tsuensis* productivity of copepod cultures were closely related to chlorophyll *a* <10µg L^{-1} concentration and hence to fertilisation rate, however excessively high levels of chlorophyll *a* resulted in a decline in the production of copepodids and adults.

Not until the experiments had been run, and DAC facility down time permitted the opportunity to analyse data was it realised a mistake had been made in the calculation of the algal rations. The correct calculated algal carbon concentrations and correct algal cell densities calculated to achieve a 500 µgC L^{-1} ration are listed in Table C6.4 with the true carbon concentration of the experimental diets assessed are detailed in Table C6.5.

Table C6.4: Corrected algal cell carbon content (pgC $cell^{-1}$), cell densities required to achieve a ration of 500 µgC L^{-1}.

	pgC $cell^{-1}$	500 µgC L^{-1}
Cryptomonas	19.7	2.5×10^4
Heterocapsa	130	3.8×10^3
Isochrysis	19.7	2.5×10^4
Nitzschia	13.1	3.8×10^4
Rhodomonas	41.6	1.2×10^4
Tetraselmis	129	3.9×10^3

Table C6.5: Algal species cell densities used to assess the effect of algal diet cell carbon concentration on *Acartia* productivity.

Experimental algal cell carbon concentration (µgC L^{-1})	*Isochrysis* algal cell carbon (µgC L^{-1})	*Heterocapsa* algal cell carbon (µgC L^{-1})	*Rhodomonas* algal cell carbon (µgC L^{-1})	*Tetraselmis* algal cell carbon (µgC L^{-1})	Actual algal cell carbon (µgC L^{-1})
200	26	4	27	41	97
400	49	7	54	84	194
800	99	14	108	168	389
1,600	197	27	212	335	772
3,600	394	56	416	671	1537
6,400	788	111	874	1290	3062
12,800	1576	221	1706	2709	6212

The diet treatment algal cell carbon concentrations applied were in effect half the intended algal cell carbon ration. The intended even distribution of algal species carbon contribution was also heavily skewed toward *Tetraselmis* reducing the contributions of *Heterocapsa* and *Rhodomonas*.

C7 Medium scale algal species trial

Acartia culture productivity would appear to be hampered in small volumes, and the density of cultures observed has generally been less than that achieved for *Tisbe* and *Apocyclops* cultures maintained under similar conditions. Attempts to obtain fine demographic information using the individual culture techniques adopted with the *Tisbe* and *Apocyclops* were also unsuccessful.

The following trial was conducted to test:

- the suitability of 10L culture volumes to the maintenance of Acartia populations,
- an alternative population sampling method, and
- the influence of microalgal species diet composition on Acartia culture productivity.

C7.1 Materials and methods

Thirty-two 20 L white plastic buckets were arranged around the edge of a partially filled 7 m^3 tank. Constant water flow through the tank acted as a water bath providing a buffer against diurnal temperature variations. The culture vessels were situated outdoors, exposed to filtered natural sunlight and natural photoperiod (14L:10D).

A total of 40 L of each of the eight treatment diets were prepared as detailed in Table C7.1 using algae cultured as described in Section 3.2 in combination with 0.1 µm filtered seawater at 35 ‰.

A 10 L volume of each of the eight treatment diets was distributed to each of four replicate cultures randomly positioned within the 7 m^3 water bath (Figure C7.1). To each of the thirty-two culture vessels was added late stage *Acartia* copepodids, which had been rinsed in 1 µm filtered, ultra-violet sterilised seawater at 35 ‰, to achieve a final density of 100 L^{-1}.

Table C7.1: Details of the eight treatment diets assessed in the medium scale algal diet trial conducted with *Acartia*. Iso – *Isochrysis*, Rho – *Rhodomonas* and Tet – *Tetraselmis*.

Diet	*Isochrysis* (cells mL^{-1})	*Rhodomonas* (cells mL^{-1})	*Tetraselmis* (cells mL^{-1})
Control	0	0	0
Iso	$1x10^5$	0	0
Rho	0	$6x10^4$	0
Tet	0	0	$6x10^4$
Iso & Rho	$5x10^4$	$3x10^4$	0
Iso & Tet	$5x10^4$	0	$3x10^4$
Rho & Tet	0	$3x10^4$	$3x10^4$
Iso, Rho & Tet	$2x10^4$	$2x10^4$	$3x10^4$

Each morning (08:00 hours) temperature, salinity, dissolved oxygen, pH and light levels were monitored prior to sampling.

The culture vessels were sampled using a device which collected a vertical section of the water column. The sampling device consisting of a 600 mm length of 40 mm PVC fitted with a ball valve at one end, and 5 mm mesh screen over the opposite end (Figure C7.2). The sampling device was lowered into each culture vessel with the ball valve in the open position. When the mesh-covered end of the device was on the bottom of the culture vessel the ball valve was closed, and the sampling device lifted out of the culture. The mesh screen helped maintain surface tension and keep the sample in the device until the ball valve was opened with the mesh-covered end over the appropriately labelled sample container. The process was repeated five times in each culture vessel with the column sampled at the four compass points and from the centre yielding a sample volume of 500 mL. A 50 mL volume of a 1:1 mixture of formalin and glycerol was added to each sample to preserve the *Acartia* life stages for analysis at a later date.

The 500 mL sample removed was replaced with the appropriate algae ration the salinity and volume of which was adjusted to maintain consistent culture volume and salinity.

Temperature, salinity, dissolved oxygen, pH and light levels were also monitored each afternoon (16:00 hours) to obtain information incorporating diurnal fluctuations and the effects of the 500 mL of new culture medium.

The trial was conducted over a 16 days. On day 8, all cultures experienced a 95% water exchange achieved by gently passing the entire culture contents over a submerged 44 µm mesh and returning the retained copepodids to the same culture vessel filled with fresh media of the appropriate algal composition.

The trial was terminated on day 16 at which time all remaining *Acartia* were screened over a 36 µm-mesh screen and preserved in a 1:1:8 mixture of glycerol, formalin and culture medium.

Sample & Statistical Analysis

Samples and subsequent data were treated in the same manner as described in C3.1.

Figure C7.1: The medium scale algal diet trial conducted in 10L volumes in 20L vessels maintained in a large water bath (7 m^3 tank filled to 1 m^3) under a shade structure at the Darwin Aquaculture Centre, Stokes Hill Wharf.

Figure C7.2: Water column sampling device consisting of a 600 mm length of 40 mm PVC (b) fitted with a ball valve (a) at one end, and 5 mm mesh screen over the opposite end (c). The sampling device is lowered into each culture vessel with the ball valve in the open position. When the mesh-covered end of the device was on the bottom of the culture vessel the ball valve was closed, and the sampling device lifted out of the culture.

C7.2 Results

Algal diet composition exerted a significant influence on *Acartia* culture productivity as indicated by the number of individuals present over the sixteen-day duration of the trial ($p<0.01$). Repeated measures ANOVA conducted on square root transformed data identified *Rhodomonas*, *Isochrysis* plus *Rhodomonas*, and *Isochrysis* plus *Tetraselmis* algal combinations to be the most productive over the sixteen day trial with an average 55 ±4.7 *Acartia* L^{-1} (Figure C7.3). Unfed cultures differed significantly from all other cultures with a mean population of 6.8 individuals L^{-1} compared to an average 26 individuals L^{-1} developing in cultures fed *Isochrysis*, *Tetraselmis* or *Rhodomonas* plus *Tetraselmis*. The effect of time was also significant ($p<0.05$), as was the interaction between algal diet composition and culture age ($p<0.05$).

Culture densities were observed to peak on day three of the trial at 115 ±15 *Acartia* L^{-1}, although the total number of *Acartia* recorded from cultures on days four and six were not significantly different (Figure C7.4a). Copepod numbers were observed to decline significantly on day seven to 22 ±3.8 *Acartia* L^{-1}. The 100% culture medium exchange conducted on day eight was followed by a slight decline in numbers with an increase observed on the final sixteenth day of the trial.

Over the sixteen days of the trial, rotifer contamination became evident, numbers experiencing a significant increase over time from zero to 220 L^{-1} on day 8. The 100% culture medium exchange on day 8 reduced rotifer numbers by 95%, however numbers recovered to day eight levels within three days, increasing 300% by day 15 reaching densities of 600 L^{-1}. No significant relationship between rotifer number and *Acartia* density was identified, however, an inversely proportional trend is apparent (Figure C7.4b).

Samples collected the first day of the trial indicated that the inoculum density was in fact 39 ±2.3 *Acartia* L^{-1} and not necessarily the intended 100 L^{-1}. No significant difference was identified between algal diet treatments one day after inoculation (Figure C7.5 Day 1).

Algal diet composition had a significant effect on culture productivity by day 8 with *Rhodomonas*, *Rhodomonas* plus *Tetraselmis* and the mixture of *Isochrysis*, *Rhodomonas* and *Tetraselmis* increasing in numbers to 48 ±6.2, 56 ±20.0 and 79 ±24.9 *Acartia* L^{-1} respectively. These three algal diets were not significantly different from each other, but were significantly more productive than all other algal diet combinations tested with 0.5 *Acartia* recorded from unfed cultures, with *Isochrysis, Tetraselmis* and *Isochrysis* plus *Rhodomonas* fed cultures producing 10 ±4.0 *Acartia* L^{-1} (Figure C7.5 Day 8).

By the sixteenth day of the trial only *Rhodomonas* plus *Tetraselmis* fed cultures maintained population densities above the inoculum density producing 76 ±22.0 *Acartia* L^{-1} (Figure C7.5 Day 16). The variation in culture densities within each algal treatment diet resulted in all cultures fed *Rhodomonas* as a component of their diet yielded higher population densities, however they did not differ significantly from *Isochrysis*, *Tetraselmis* and *Isochrysis* plus *Tetraselmis* fed cultures producing an average 25 ±6.8 *Acartia* L^{-1}.

Light intensity did not vary significantly between diet treatments over the duration of the trial as determined by repeated measures ANOVA ($p>0.05$). The overall light intensity was 23 lux with an associated range from 17 to 29 lux.

Salinity and temperature levels across cultures were also statistically the same with mean salinity of 35.4‰ and temperature of 29.4°C over the duration of the trial associated with rages of 35 ‰ to 37.5 ‰ and 27.0°C to 30.4°C.

In contrast the pH and dissolved oxygen levels recorded for the *Acartia* culture media differed significantly between diet treatments ($p<0.01$).

A difference of 0.4 mgO_2 L^{-1} between *Isochrysis* fed cultures and the unfed control cultures and the cultures fed a diet of *Rhodomonas* and *Tetraselmis* (R+T) resulted in the statistically significant difference. The dissolved oxygen levels recorded for all other diet treatment cultures did not differ over the sixteen days of the trial (Table C7.2).

Similarly an overall difference in means of less than 0.1 pH units resulted in statistically different culture media pH levels between *Isochrysis* fed cultures and all other treatment diets (Table C7.3).

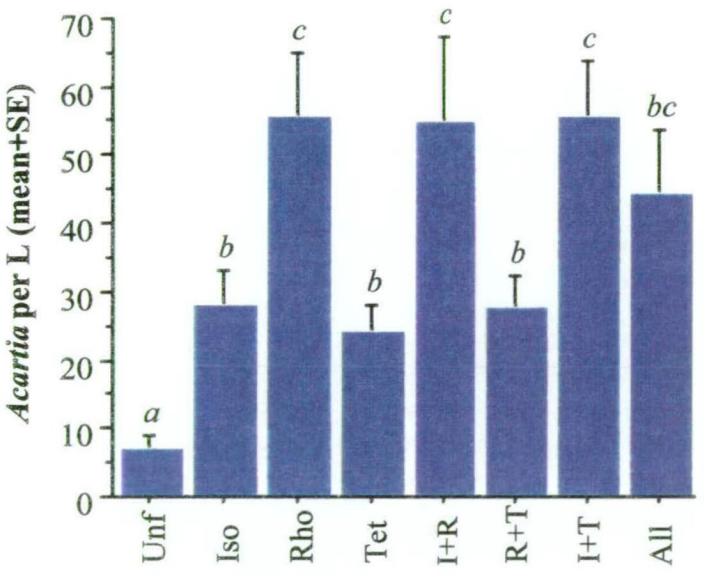
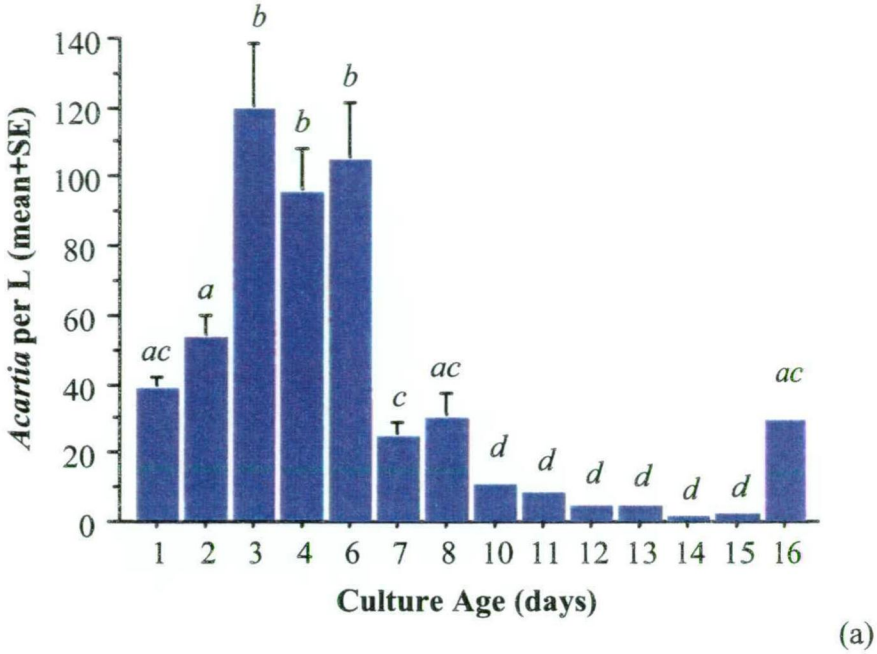


Figure C7.3: Influence algal diet on *Acartia* culture productivity as indicated by the total number of individuals developing over sixteen days after inoculation with copepodids at 100 L^{-1}. Italicised superscripts indicate significantly different ($p<0.01$) means as identified by repeated measures ANOVA of square root transformed data in conjunction with Scheffe's multiple means comparison. Unf – seawater, Iso – *Isochrysis*, Rho – *Rhodomonas*, Tet – *Tertaselmis*, and All – a mixture of all three algae.



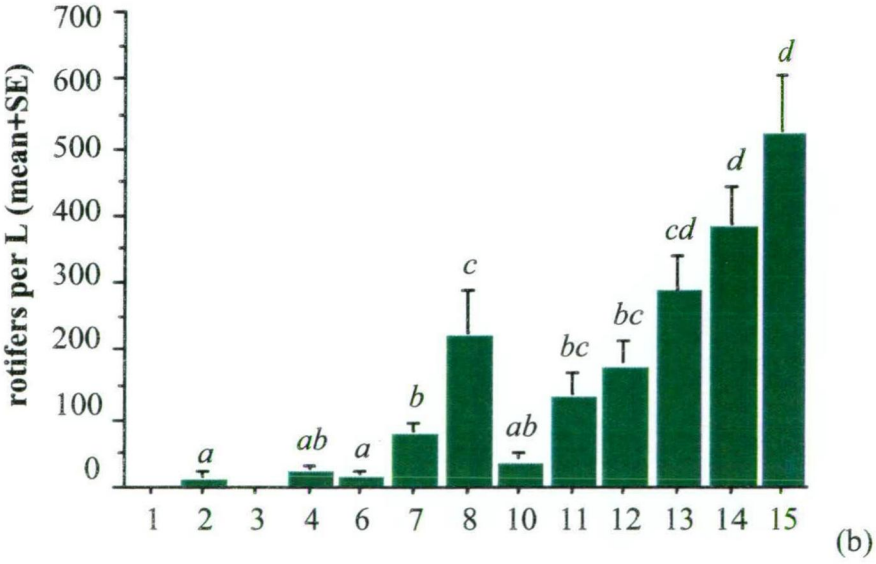


Figure C7.4: Influence time on *Acartia* culture productivity as indicated by the total number of individuals developing over sixteen days after inoculation with copepodids at 100 L^{-1}. Italicised superscripts indicate significantly different ($p<0.01$) means as identified by ANOVA of square root transformed data in conjunction with Scheffe's multiple means comparison.
Unf – seawater, Iso – *Isochrysis*, Rho – *Rhodomonas*, Tet – *Tertaselmis*, and All – a mixture of all three algae.

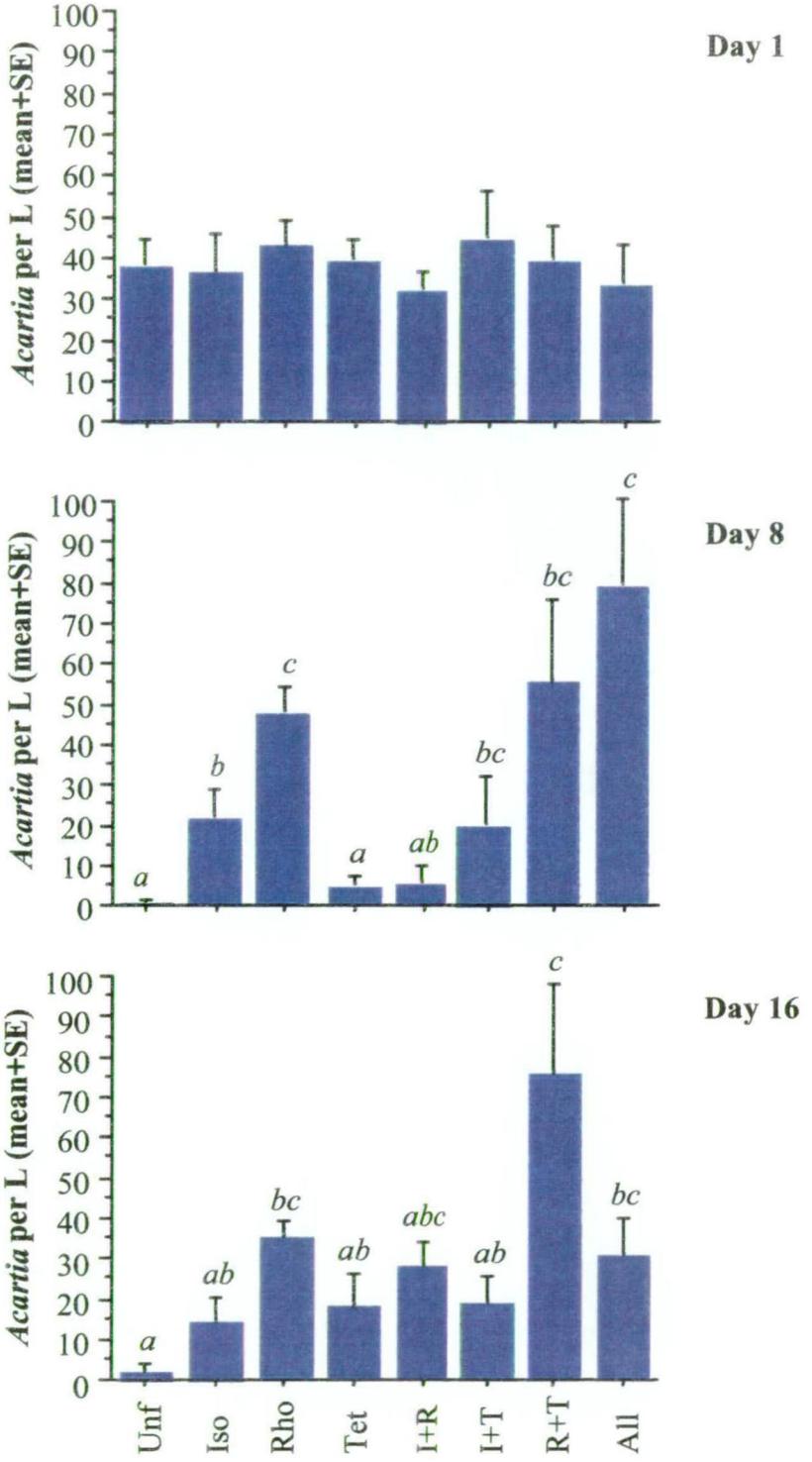


Figure C7.5: The influence of diet on the productivity of *Acartia* cultures as determined by the total number of individuals present after 1, 8 and 16 days in 10 L cultures maintained at 29 °C and 35 ‰ after inoculation at 100 copepodids L^{-1}. Italicised superscripts indicate significant differences as determined by ANOVA of square root transformed data and Scheffe's multiple means comparison ($p<0.01$). Iso, I – *Isochrysis*, Rho, R – *Rhodomonas*, Tet, T – *Tetraselmis*, All – all three algae.

Table C7.2: The mean dissolved oxygen (mean mgO_2 L^{-1} ±SE) recorded over the sixteen days of *Acartia* culture fed seven algal diet combinations.

Diet	Mean	Min	Max	$p<0.05$
Unfed	6.17 ±0.06	5.4	8.4	*a*
Isochrysis	6.59 ±0.10	5.3	10.2	*b*
Rhodomonas	6.31 ±0.08	5.3	9.4	*ab*
Tetraselmis	6.18 ±0.08	5.4	8.6	*ab*
I+R	6.32 ±0.08	5.2	8.9	*ab*
R+T	6.15±0.06	5.2	7.8	*b*
I+T	6.36 ±0.10	5.2	9.5	*ab*
All	6.29 ±0.07	5.4	8.4	*ab*

Table C7.3: The mean pH (mean ±SE) recorded over the sixteen days of *Acartia* culture fed seven algal diet combinations.

Diet	Mean	Min	Max	$p<0.05$
Unfed	8.11 ±0.01	7.86	8.30	*a*
Isochrysis	8.10 ±0.02	7.87	8.49	*b*
Rhodomonas	8.19 ±0.02	7.87	8.49	*a*
Tetraselmis	8.12 ±0.01	7.88	8.39	*a*
I+R	8.11 ±0.02	7.89	8.45	*a*
R+T	8.13 ±0.02	7.87	8.45	*a*
I+T	8.15 ±0.02	7.90	8.49	*a*
All	8.13 ±0.02	7.88	8.43	*a*

C7.3 Discussion

The 10 L static cultures maintained with a daily algal addition did not meet productivity expectations. The unexpectedly low *Acartia* densities may be in part due to competition with rotifers for food, and the negative impact of rotifer metabolites on copepod reproduction. Due to dismantling of facilities the constant temperature room was unavailable, the cultures maintained outdoors where rotifer contamination was possible through aerosol transfer. The use of the same monitoring and sampling equipment between treatments may also have facilitated rotifer transfer despite rinsing of equipment in fresh water between replicates. Aerosol transfer from adjacent mudcrab larval rearing trials may be the source of contamination.

Future trials assessing the influence of diet on *Acartia* productivity would benefit from a more controlled environment and the use of flow-through systems.

Rhodomonas and *Tetraselmis* demonstrated their ability to support productive *Acartia* populations producing 80 *Acartia* L^{-1} after sixteen days compared with the other diets, which maintained populations half as dense. The value of *Rhodomonas* to the long-term culture of tropical *Acartia* in Australia has been supported by work conducted by the Queensland counterpart of DAC (Gail Semmens, QDPI, *pers. comm.*).

In terms of the initial aims of the trial it is difficult to extrapolate regarding the suitability of static 10L culture volumes. Initial *Acartia* population increases prior to rotifers blooming suggest that the larger volume may be beneficial to *Acartia* culture.

The alternative sampling method shown to work well in culture volumes measured in m^3 may have been appropriate had *Acartia* densities continued to increase.

Acartia populations fed diets containing *Rhodomonas* exhibited the most consistent increase in density despite any confounding influences of rotifers.

C8 Survival of golden snapper larvae in small volume aquaria as influenced by larval stocking density

Survival of golden snapper larvae was reportedly poor in small volumes less than 1L (Glenn Schipp, DAC, *pers. comm.*). The need for replication of treatments in the assessment of larval performance favours the use of small volume experimental systems to facilitate the most efficient use of available resources investigating larval performance when fed copepod life stages as opposed to rotifers and *Artemia*. The following trial was designed to assess the influence of larval survival in 3.5 L aquaria as affected by larval stocking density.

C8.1 Materials and methods

Fifteen aquaria were set up as described in Section 3.2.3 Figure 3.2.4 and inoculated under low light conditions late in the evening with golden snapper larvae at 1 day post-hatch (dph) using a 50 mL beaker. The larvae used were obtained from a first night spawning. Three replicate aquaria were inoculated at each of the five densities: 5, 10, 20, 35 or 50 larvae L^{-1}.

Larvae were still visible in the water column of all aquaria on the second and third days of the trial, but numbers appeared to diminish over following days, with no larvae visible on day 5. All aquaria were subsequently emptied through a 44 µm mesh screen and checked for the presence of larvae.

C8.2 Results

Larval golden snapper survival is poor in 3.5 L aquaria. No live *L. johnii* larvae were found at the end of the four day trial. Both calanoid and cyclopoid adult copepodids, including a number of ovigerous cyclopoids, were present in the aquaria. All concurrent trials stocked with golden snapper larvae from the first night of spawning exhibited poor survival.

Salinities ranged from 30 to 32.5 ‰, temperature 29.8 to 31.1 °C, pH fell from 8.2 to 7.9, and ammonia levels increased to 0.035 mg unionised ammonia L^{-1} with only traces of nitrite detected. Algae remained reasonably constant at around 10^4 cells mL^{-1}. Live food densities were maintained between 2 and 5 *Acartia* nauplii mL^{-1} through the addition of further quantities of live food as necessary during the trial.

C8.3 Discussion

The relative lack of success achieved with these trials may be largely attributable to larval quality as all concurrent trials in larger systems experienced similarly high larval mortalities. Ammonia levels during the trial were less than the 0.01 mg L^{-1} as recommended by Lim et al. (1985).

The emaciated state of the golden snapper larvae suggests feeding had not been initiated since their mouths opened late during the afternoon of 2 dph. Live food was not presented until 8 pm on the second day after hatching, possibly 20 hours after the commencement of exogenous feeding. Similar poor survival rates were obtained with larvae presented food 6 hours earlier, which would imply that food deprivation was not the sole factor affecting survival of the golden snapper larvae.

The inclusion of an unfed control would have provided some indication as to the influence of any perceived delay in the presentation of live food. Further confounding factors may have related to aquarium design suitability. These aspects of experimental design were taken into consideration during subsequent trials.

C9 Barramundi survival as influenced by live food type

Initial trials conducted using barramundi larvae fed rotifers and *Acartia* nauplii to investigate the potential to conduct short-term feeding trials with first-feeding larvae were met with difficulties in timing with respect to the rapid digestion of food. Unlike temperate fish species, first feeding larvae were able to digest prey items beyond recognition within a 4 hour period at the commencement of exogenous feeding in contrast to the 6 hours-plus observed with temperate species.

The more hardy nature of the barramundi larvae, attributable to a less sensitive lateral line of barramundi lacking the prominent cupulae evident on the neuromasts of the golden snapper (Doi & Singhagraiwan, 1993), rendered the completion of small scale trials more likely to yield results. Bagarinao (1986) identified the prominent cupulae present on rabbit fish larvae as the primary reason behind their intolerance of handling in comparison to the observed hardiness of barramundi.

Recognising the logistical difficulties and the limited information able to be supplied by detailed work, it was decided to attempt a small scale larval performance trial comparing growth rates observed for larvae when reared on various live food species diet combinations.

A short term performance trial was conducted over 5 days in 250 mL aquaria to assess the performance of barramundi larvae fed one of six diets comprising either rotifers, *Artemia*, *Acartia* nauplii, rotifers followed by *Artemia*, a mixture of the three live foods, and an unfed control.

C9.1 Materials and methods

Thirty-six cylindrical 250 mL plastic aquaria were washed in freshwater and air-dried prior to being filled with 200 mL of 1 µm filtered seawater at 35 ‰. Six replicate aquaria were inoculated with each of the six live food combinations: 1) rotifers, 2) *Acartia* nauplii, 3) Artemia, 4) rotifers and *Artemia*, 1:1, 5) a mixture of rotifers, *Acartia* nauplii and *Artemia*, 1:1:1, or 6) left unfed as a control.

Live foods were maintained and prepared as previously described in Section 3.4.2. The final density of live food organisms (LFOs) was 10 individuals mL^{-1} for all treatments. To ensure consistency between treatments the live foods were harvested from their culture

vessels, rinsed and resuspended in clean seawater at a density of 90-100 individuals mL^{-1}. A 30 mL volume of the appropriate live food was then introduced to the aquaria using a 50 mL syringe.

Twenty 2 dph barramundi larvae were individually transferred using a wide bore plastic pipette (Ø 8 mm) to each of the thirty-six aquaria. The larvae were allowed to feed at a temperature of 28 to 30°C under a photoperiod regime of 13 L:11D. Barramundi larvae were individually transferred daily to new aquaria containing clean 1 µm filtered seawater at 35 ‰ and the appropriate recently harvested live food at a final density of 10 mL^{-1}. Larvae were transferred daily to alleviate potential water quality deterioration, maintenance of live food densities and determination of daily survival rates. Salinity and temperature were recorded daily.

Survival rate data was unable to be transformed to not meet the assumptions underlying analysis of variance and were subject to Kruskal-Wallis *k*-sample analyses in conjunction with Tukey's multiple means comparison test.

C9.2 Results

The influence of live food offered on larval survival was observed to be age dependent. No significant difference was evident in survival rates exhibited by 2 dph and 3 dph larvae between the six diet treatments, exogenous feeding having commenced late on day two post-hatch. However, highly significant differences ($p<0.001$) were identified for larvae of 4 dph and 5 dph (Figure C9.1).

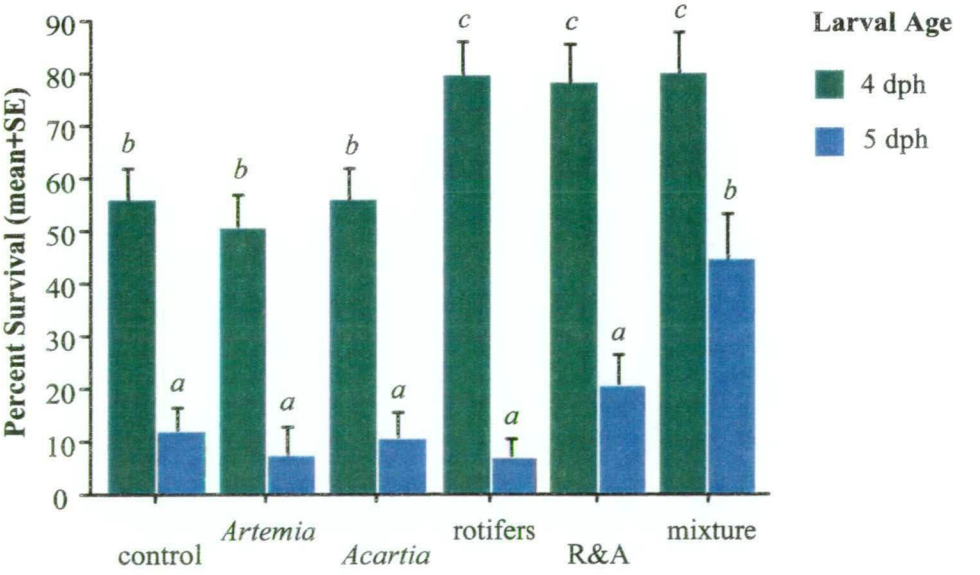


Figure C9.1: Barramundi survival at 4 and 5 days post-hatch (dph) when fed one of six diets including an unfed control and five live food combinations: *Artemia* nauplii, *Acartia* nauplii (stages NI to NVI), rotifers (*Brachionus rotundiformis*), a mixture of *Artemia* and rotifers (R&A), and the mixture a combination of rotifers, *Artemia* and *Acartia*. All live food combinations resulted in a final density of 10 organisms mL^{-1}. Italicised superscripts indicate significant differences identified by Kruskal-Wallis *k*-test and Tukey's multiple means comparison test.

At 4 dph two groups are evident. The first comprising unfed controls, *Artemia* and *Acartia* fed treatments averaging 54 ±0.6% (mean ±standard error), the second encompassing those fed rotifers, a mixture of rotifers and *Artemia*, and those fed a mixture of all three live food types averaging 79 ±0.2%.

The survival rates of larvae from all aquaria were significantly higher at 4 dph than at 5 dph. At 5 dph the average survival rate dropped from 66.7% to 17.1%. Only the larvae fed a mixed diet maintained a similar survival rate decreasing to only 44.7% compared with the average of 11.4% for the remaining 5 diet combinations.

The temperature and salinity recorded during the trial averaged 28.5 °C and 36 ‰ respectively. The photoperiod for the duration of the trial was set at 13 L:11D in the constant temperature room.

C9.3 Discussion

The inclusion of *Acartia* nauplii in the diet of barramundi nauplii would appear to exert a beneficial influence on larval barramundi survival at 5 dph.

The point of no return, when 50% mortality occurs even when live food is offered again (Bagarinao, 1986; Koven et al., 1999), for barramundi is coincident with complete yolk sac absorption occurring at approximately 3 dph depending on water temperature (Bagarinao, 1986; Kohno et al., 1994). Differences in survival between larvae in aquaria exposed to different live food combinations beyond 3 dph may therefore be attributed to differences in larval barramundi diets. The greater survival of barramundi in copepod-supplemented aquaria may therefore be attributed to the presence of *Acartia* nauplii.

The feeding of mixed diets containing copepods and rotifers has been associated with improved growth and survival for a number of species including the Western Australian dhufish and pink snapper (Payne et al., 2001). Western Australian dhufish larvae presented a mixed diet (50% rotifer with 50% calanoid *Gladioferens imparipes* nauplii) yielded larvae of 11 mm in length nine days in advance of dhufish larvae fed solely rotifers. Copepod supplemented larvae also exhibited a 550% better larval survival rate. Similarly pink snapper larvae reared on rotifers only were significantly smaller and exhibited a slower rate of growth then those fed calanoid nauplii for six days of feeding prior to being fed rotifers (Payne et al., 2001).

Appendix D
Journal reprint (has been removed for copyright reasons)
Aquaculture 174:81-88 (1999)
A method for hatchery culture of tropical calanoid copepods, Acartia spp.
Glenn R. Schipp, Jerome M.P. Bosmans, Andria J. Marshall

Appendix E
Preliminary Fatty Acid Analyses

E1 Preliminary fatty acid analyses of candidate live food organisms

E1.1 Introduction

During the initial stages of research into alternative live food organisms, copepods were but one option. During the first few months of research a number of zooplankton species were collected and maintained in culture including amphipods, copepods and mysids.

Dietary lipid fatty acid composition has been recognised as one of the fundamental areas with marine larvae exhibiting an absolute requirement for long chain polyunsaturated fatty acids, in particular the n-3 family members eicosopentanoic acid (EPA, 20:5n3) and docosahexanoic acid (DHA, 22:6n3). These fatty acids are crucial to membrane stability and function, with DHA of great importance in the makeup of neural and sensory cell membranes. Developing fish larvae comprise a disproportionately large component of neural tissue and DHA and EPA are thus of principal importance. More recently the essential role arachidonic acid (AA) has been recognised (Craig et al., 1995; Robin, 1995; Nanton & Castell, 1999; Koven, 2001).

Samples of amphipods, mysids, and copepods were subject to preliminary fatty acids analysis to obtain some indication as to the suitability of the invertebrate species as live food organisms for larviculture in terms of the relative composition of the essential fatty acids AA, DHA and EPA.

E1.2 Materials and methods

Samples were obtained as detailed in Table E1.2.1 and carefully screened to remove any particulate contaminants. At least 200 mg wet weight of fresh material was collected in a manner so as to ensure they were free of contamination from plastics, greases, flesh contact or foreign matter. Collected samples were rinsed in three volumes of technical grade 0.4M ammonium formate prior to transfer to labelled, preweighed KIMAX tubes. Wet weights of the samples were recorded prior to being stored –80 °C for a maximum period of 3 months. Samples were processed at the CSIRO marine laboratories, Hobart, Tasmania, with the lipid fraction extracted using the modified Bligh & Dyer technique and the total fatty acid methyl esters analysed by gas chromatography-mass spectrometry (Volkman et al., 1989). Fatty acid identifications were confirmed by comparing mass spectra and retention data with those previously reported and with those obtained from commercial standards.

Table E1.2.1: Preliminary results from samples subject to fatty acid analyses at CSIRO Marine Laboratories, Hobart, Tasmania.

Sample	Source details
Tisbe	Ovigerous females and nauplii collected from cultures maintained at Launceston, Tasmania.
Apocyclops	Ovigerous females sampled 24 hours after collection from pond zooplankton at Barramundi Farms NT.
Artemia	Prime® cysts sampled within 24 hours of hatching, unenriched.
amphipods	Collected from decomposing seaweed on the beach at Low Head, Tasmania.
mysids	*Mysis* species collected from Waubs Bay, Bicheno Tasmania.

E1.3 Results

Significant differences in the relative levels of the three target fatty acids were detected between the six sample types collected. *Tisbe* and *Apocyclops* samples exhibited DHA:EPA ratios in excess of 1.0 (Tables E1.3.1 and E1.3.2), a characteristic considered desirable in live foods (Tucker, 1992), as did freshly collected mysids (Table E1.3.3). The DHA:EPA ratio of *Tisbe* nauplii was approximately twice that of ovigerous individuals.

Table E1.3.1: Indicative essential fatty acid percent composition (mean % ±standard error) for ovigerous *Apocyclops dengizicus* and unenriched *Artemia* nauplii.

Fatty Acid	*A. dengizicus*	*Artemia*
AA	2.9 ±0.06	0.5 ±0.00
EPA	3.6 ±0.07	3.7 ±0.79
DHA	18.3 ±0.35	0.1 ±0.02
DHA:EPA	5.1 ±0.03	0.04 ±0.00

Table E1.3.2: Indicative essential fatty acid composition (%) for ovigerous *Tisbe* and nauplii of *Tisbe*.

Fatty Acid	ovigerous *Tisbe*	*Tisbe* nauplii
AA	4.4	2.7
EPA	9.0	6.6
DHA	10.3	17.0
DHA:EPA	1.1	2.6

Table E1.3.3: Indicative essential fatty acid composition (%) for amphipods, mysids 1 collected fresh and mysids 2 sampled after three months in culture.

Fatty Acid	amphipods	mysids 1	mysids 2
AA	4.5	1.4	0.9
EPA	13.4	17.6	11.0
DHA	13.7	19.6	5.9
DHA:EPA	0.3	1.1	0.5

E4 Discussion

Only three of the six zooplankton types exhibited promising fatty acid compositions. Mysids were not investigated further as their primary food stuff was *Artemia* combined with a minimum size in excess of 200 µm being unsuitable for first feeding finfish larvae.

In contrast both copepod species sampled exhibited favourable DHA:EPA ratios and life stages of an appropriate size suitable for use a live foods for first feeding larvae.

Further analysis of live foods was not undertaken due to time and financial constraints, however these preliminary results agree with published literature espousing the nutritionally replete fatty acid profile of copepods in general. *Eurytemora* and *Tisbe furcata* exhibited natural ratios of 2 and 2.2 respectively compared to the maximum of 1.3

achieved with *Artemia* enriched with fish oils high in DHA and EPA (Bell et al., 2001). Norsker & Støttrup (1994) reported DHA:EPA ratio of 2.8 approximately that reported here for cultured Tasmanian *Tisbe* nauplii.

Evjemo et al. (2001) suggested that copepods should be used as a reference point from which to define the lipid content of *Artemia* species and rotifers post-enrichment following a study of wild caught zooplankton which revealed *n*-3 HUFA to be almost twice that of enriched, cultivated species such as rotifers and *Artemia*.